Proteomic Analysis of Normal and Cancer Cervical Cell Lines Reveals Deregulation of Cytoskeleton-associated Proteins

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Abstract. Background: Both HPV-positive and -negative cervical cancers are primarily associated with features of cell cycle and cytoskeletal disruption; however, the actual biological processes affected remain elusive. To this end, we systematically characterized the intracellular proteomic profiles of four distinct and informative cervical cell lines. Materials and Methods: Cell extracts from a normal cervical (HCK1T) and three cervical cancer cell lines, one HPV-negative (C33A), and two HPV-positive, SiHa (HPV16+) and HeLa (HPV18+), were analyzed by 2-dimensional electrophoresis and differentially expressed proteins were identified by MALDI-TOF mass spectrometry, while differential expression was confirmed by western blot analysis. Results: In total, 113 proteins were found differentially expressed between the normal and the cervical cancer lines. Bioinformatics analysis revealed the actin cytoskeleton signaling pathway to be significantly affected, while up-regulation of cofilin-1, an actin depolymerizing factor, was documented and further validated by western blotting. Furthermore, two-way comparisons among the four cell lines, revealed a set of 18 informative differentially expressed proteins. Conclusion: These novel identified proteins provide the impetus for further functional studies to dissect the mechanisms operating in the two distinct pathways of cervical carcinogenesis.

Cervical cancer represents the fourth most common and fatal form of cancer among women worldwide (1). More than 90% of cervical cancer cases arise as a consequence of a human papilloma virus (HPV) infection. Currently, 210 different HPV types have been officially recognized (2), and – based on their carcinogenic potential – have been classified either as low-risk HPV types, or as high-risk and potentially carcinogenic (3). Of the high-risk group, the five most common HPV types include HPV 16, 18, 45, 30, and 33, while types 16 and 18 alone, account for about 70% of all cervical cancer cases (4).

In the early phase of cervical carcinogenesis, HPV initially infects the proliferating cells of the basal layer of the cervical stratified epithelium through abrasions of the mucosal epithelium. Following infection, the HPV genome remains in the form of nuclear extrachromosomal episome, and due to the repression of the viral E6 and E7 oncoprotein synthesis, the viral DNA replication occurs at very low levels (5). Following the gradual differentiation of the basal cells and their migration to the upper layers of the epithelium, an increased expression of E6 and E7 oncoproteins occurs, leading to inhibition of apoptosis, while the viral genome is replicated further. At this point, structural proteins and mature viral particles are produced, and the shed virus can then initiate a new infection. These
infections resolve within 1-2 years, but they can last up to several decades. However, very few of them are persistent and gradually progress to cancer (6), reflecting a dynamic interplay of viral mechanisms of immune escape and suppression of the negative regulators of the cell growth, combined with defects of cellular response by the host (5). The final events of the neoplastic transformation are associated frequently (45-80%) with the integration of the HPV DNA into the host genome and the ensuing overexpression of E6 and E7 oncoproteins, leading to further proliferation of the transformed cells.

Since both HPV-positive and HPV-negative cervical cancers are primarily associated with features of cell-cycle and cytoskeletal disruption (5), the precise elucidation of the contribution of the putative individual oncogenic drivers induced by the presence or the absence of HPV in cervical cancer is imperative. Therefore, utilization of informative cell lines with or without the HPV genome, can provide valuable insights on these mechanisms. To this end, our group has initiated a comprehensive approach to elucidate the specific oncogenic drivers operating in cervical cancer, both at the transcriptional (7) and the proteomic level (8). In these studies, we have identified for the first time, four novel transcription modules (7), involved in cervical cancer, exhibiting synergy between groups of transcription regulators, while certain modules were annotated to specific biological processes, such as cell cycle, apoptosis, transcription and development. In our recent study (8), by employing proteomic approaches on the secretome of four informative cervical cell lines, we have identified 67 differentially expressed proteins, displaying mainly catalytic, binding or structural molecule activity, while bioinformatics analysis identified the transcription factor NRF2 as an important regulator of differentially expressed proteins in the cancer cell lines. Thus, such comparative proteomic approaches employing cervical cell lines, represent a valuable tool to further explore the precise mechanisms involved in viral infection and protein dysfunction interplay that can lead to cervical carcinogenesis (5, 6). Furthermore, a review (6) of recent studies on the proteomics of cervical cancer cell lines, revealed that their major focus relies either on the variable effects of several drugs or on the modulation of a specific gene expression on their proteome composition.

Therefore, based on the above data, in the present study, we further investigated these mechanisms, by employing 2-dimensional electrophoresis (2-DE) and bioinformatics analysis, and systematically characterized the intracellular proteomic profiles of four distinct informative cervical cell lines, either with or without the presence of HPV, and identified specific biochemical similarities and differences, reflecting particular aberrant pathways of carcinogenesis, which can be eventually validated further and assessed as putative biomarkers of cervical pathology.

Materials and Methods

Cell lines culture and sample preparation. HeLa (HPV 18+), SiHa (HPV 16+) and C33A (HPV-negative) cervical cancer cell lines, were purchased from ATCC (Manassas, VA, USA) and cultured in Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS) (Gibco-Invitrogen, Waltham, MA, USA) at 37°C, 5% CO2, as previously described (9). HCKT1 cell lines were a kind offer of Tosh Kiyono (10) and were cultured as proposed (11) in Defined Keratinocyte Serum-Free Medium (SFM) (Gibco BRL, San Francisco, CA, USA) supplemented with 5 ng/ml Epidermal Growth Factor (EGF) (Gibco BRL) and 50 μg/ml of Bovine Pituitary Extract (BPE) (Gibco BRL). When the cells reached a concentration of 106 cells per ml, they were trypsinized and harvested, and the pellets were washed in Phosphate Buffered Saline (PBS) 3 times. Pellets were homogenized in 2D-Buffer (7 M Urea, 2 M thiourea, 4% CHAPS, 1% DTE) using mild sonication (water bath sonication). After centrifugation at 16,000 x g for 20 min, the total cell extract was obtained as a supernatant. Protein concentration was measured with the Bradford assay.

2D Electrophoresis. From each cell extract, 80 μg were loaded on 7-cm immobilized pH gradient (IPG) strips of pH 3-10 NL (Bio-Rad, Hercules, CA, USA) in isoelectric focusing (IEF) cell trays, following the addition of 2% Bio-Lyte 3/10 Ampholytes for isoelectric focusing. For the preparative gels only, the amount of protein loaded from each cell line was 400 μg. IEF was performed in Bio-Rad PROTEAN IEF cell for 14,000 VHR as follows. Step 1: 50 V, rapid, 14 h (active rehydration); step 2: 250 V, rapid, 30 min; step 3: 4,000 V, linear, 1 h; step 4: 4,000 V, rapid, 11,000 VHR (focusing); step 5: 100 V, rapid, 24 h (conservation). For the second dimension electrophoresis, the equilibration of the strips and the reduction and alkylation of the proteins were performed in equilibration buffer (6 M Urea, 1.5 M Tris HCl, pH 8.8, 30% Glycerol, 2% SDS) containing 0.03 M DTE for 20 min and then in equilibration buffer containing 0.136 M Iodoacetamide for 20 min. Strips were then sealed with agarose on top of a 12% SDS-polyacrylamide gel, which was run at 80 V for 10 min and then at 160 V. Following electrophoresis, the gels were fixed in 30% methanol, 10% acetic acid for 30 min, 2 h and then overnight. Visualization of the spots was achieved with silver staining. Each gel was sensitized in 0.8 mM sodium thiosulfate pentahydrate solution for 1 min, washed with ultrapure water for 1 min, stained in 12 mM silver nitrate solution for 20 min and washed again with ultrapure water for 10 sec. Visualization of the spots was performed with 50 ml of development solution (3% w/v potassium carbonate, 12.5 μl formalin-formaldehyde 37%, 5.25 μl 10% w/v sodium thiosulfate pentahydrate). When the development was completed, the gel was washed with ultrapure water for 10 sec and the stop solution (2% acetic acid) was added and left for 10 min. After two washes with ultrapure water for 10 min, the gels were scanned with a GS-800 imaging densitometer (Bio-Rad). Four 2D gels from each cell line were prepared and were used for the analysis.

Comparative analysis. The comparative analysis of the 2D gels images was performed with the PDQuest 2D-Gel analysis software v.8.1.0 (Bio-Rad). The individual protein spot quantity was normalized with the total optical density of the gel. All protein spots with a cancer/normal ratio <0.5 or >2 were considered as differentially expressed and were included for further analysis.
Spot preparation and protein identification. The spots were picked manually from the corresponding preparative 2D gels, which were stained with Coomassie Colloidal Blue overnight, placed in 96-well plates, and 100 μl of destaining solution (40% ACN, 50 mM ammonium bicarbonate) was added in each spot. The plates were shaken for 15 min and this step was repeated three times. Then, the spots were washed with 100 μl ultrapure water for 5 min, reduced with 100 μl of 10 mM DTE dissolved in 100 mM ammonium bicarbonate, pH 8.5 for 10 min and alkylated with 100 μl of 54 mM iodoacetamide, dissolved in 100 mM ammonium bicarbonate, pH 8.5 for 10 min in the dark. The spots were washed with 100 μl of 100 mM ammonium bicarbonate, pH 8.5 for 5 min and then dried in a Savant SpeedVac™ concentrator (Thermo Fisher Scientific, Logan, UT, USA). Finally, 3 μl of 10 ng/μl trypsin in 10 mM ammonium bicarbonate was added and left overnight. The products of tryptic digestion were extracted from the gel by the addition of 5 μl of extraction solution (50% ACN, 0.1% TFA) for 30 min. Then, 1 μl of peptides from each spot were mixed on a stainless steel MALDI target plate with 1 μl of matrix solution (50% v/v ACN, 0.1% TFA v/v, 0.7% v/v α-cyano-4-hydroxycinnamic acid) containing the peptides des-Arg-bradykinin, 904.4681Da (Sigma-Aldrich Corp., St. Louis, MO, USA), and the adrenocorticotropic hormone fragment 18-39, 2465.1989Da (Sigma-Aldrich), as internal standards. For peptide identification, Matrix Assisted Laser Desorption Ionization-Time of Flight/Time of Flight Mass Spectrometry (MALDI-TOF/TOF MS) was performed in an Ultraflex TOF/TOF mass spectrometer (Bruker Daltonics, Billerica, MA, USA). Peak list was created with the Flexanalysis v2.2 software (Bruker Daltonics), peptide matching and protein searches were performed automatically by Mascot Server database (Matrix Science, Boston, MA, USA), signal/noise threshold ratio was set at 2.5. For peptide identification, monoisotopic masses were used and a mass tolerance of 0.0025% (25 ppm) was allowed. Cysteine carbamidomethylation and methionine oxidation were set as fixed and variable modifications, respectively. One miscleavage was allowed. The peptide masses were compared with the theoretical peptide masses of all available proteins from *Homo sapiens* using the Swiss-Prot database. The probability score with *p* < 0.05 identified by the software, was used as the criterion for the affirmative protein identification.

Bioinformatics analysis. Functional annotation of the differentially expressed proteins was performed manually using information from UniProt (http://www.uniprot.org/) and the literature. Pathway analysis was generated by QIAGEN’s Ingenuity® Pathway Analysis (IPA®, QIAGEN, Redwood City, CA, USA; www.qiagen.com/ingenuity). Ingenuity® Pathway Analysis output was manually curated in order to remove redundant terms, results unrelated to cancer biology, and pathways with fewer than three differentially expressed proteins from our dataset. Moreover, only statistically significant (*p*≤0.05, Fisher’s exact test) canonical pathways were selected.

Western blot analysis. Four 50 μg samples of cell extract dissolved in Laemli’s buffer from each cell line were loaded in 15% SDS-polyacrylamide gel after incubation at 90°C for 10 min. The gel was run at 40 V for 15 min and then at 120 V in transfer buffer (3.03 g Tris, 14.4 g Glycine, 200 ml Methanol for 1 liter total volume). The transfer was performed in transfer buffer for 2 h at 290 mA at 4°C. Then, the membrane was stained with Ponceau-S stain for 5 min, washed with ultrapure water for 5 min three times, followed by the addition of blocking solution (5% w/v non-fat dried milk in TBS-Tween 0.1% v/v) and incubation for 2 h. The membrane was washed with TBS-Tween 0.1% v/v successively for 15 min, 5 min, and 5 min, and the primary mouse antibody sc-53934 for Cofilin-1 (Santa Cruz Biotechnology, Inc., Dallas, TX, USA) was added in a 1:500 dilution and left at 4°C overnight. The next day, the three washes were repeated and the secondary sc-2005 goat anti-mouse antibody IgG-HRP (Santa Cruz Biotechnology) was added in a 1:2,000 dilution and left at room temperature for 2 h. The three washes were repeated, ECL was added and left for 1 min; its excess was removed, followed by film exposure and development.

Results

Proteomic analysis. In order to investigate the quantitative differences in protein expression resulting from malignant transformation of the cervical epithelium, the proteomic profiles of four cervical cell lines were analyzed. The four cell lines that were used were the following: HCK1T (Human Cervical Keratinocytes), a normal cervical epithelium cell line; HeLa, a cervical cancer cell line positive for HPV18; SiHa, a cervical cancer cell line positive for HPV16; and C-33A, a cervical cancer cell line negative for HPV. Four 2D gels were run for each cell line total extract. A representative gel is shown in Figure 1 for each cell line. The initial analysis was focused on the comparison between the normal cervical cell line (HCK1T) and the group of the three cervical cancer cell lines (HeLa, SiHa, C-33A). In this comparison, 43 spots, that corresponded to 48 unique proteins, were found down-regulated (cancer/normal ratio <0.5) in the cancer cell lines. Almost half of these proteins (42%), are associated to cytoskeleton and 21% of them are involved in metabolism (Figure 2). Moreover, 85 spots, that corresponded to 65 unique proteins, were found up-regulated (cancer/normal ratio >2) in the cancer cell lines. A significant percentage of these proteins are also associated to cytoskeleton (8%) and metabolism (42%) (Figure 2). Proteins that were found deregulated included cofilin-1; vinculin; vimentin; fascin; annexin A2; transgelin-2; alpha-enolase; triosephosphate isomerase; glyceraldehyde-3-phosphate dehydrogenase; peptidyl-prolyl cis-trans isomerase A; fructose-bisphosphate aldolase A; peroxiredoxins 1, 2, 5 and 6; protein DJ-1; and growth factor receptor-bound protein 2. A complete list of the differentially expressed proteins is presented in Table I.

The overlap of differentially expressed proteins among the three comparisons was examined and is presented in a Venn diagram in Figure 3. Five proteins only emerged from the comparison between the HPV-negative cervical cancer cell line (C-33A) and the normal cervical keratinocytes (HCK1T), while 13 proteins composed the HPV-positive “core”, emerging from the individual comparisons between the HPV-negative cervical cancer cell lines (HeLa and SiHa) with the HCK1T (Table II).
Bioinformatics analysis. Pathway analysis of the differentially expressed proteins between the normal (HCK1T) and the three cancer (HeLa, SiHa, C-33A) cell lines, using the Ingenuity® Pathway Analysis (IPA) software, revealed that the actin cytoskeleton signaling pathway is associated in a statistically significant manner ($p=0.029$) (Figures 4 and 5, Table III). As mentioned previously, biological function annotation also documented that a large percentage of all the differentially expressed proteins, are associated to cytoskeleton. Based on these indications, and the known biological contribution to cancer pathogenesis, proteins involved in cytoskeletal processes were further investigated. Thus, cofilin-1 (CFL1), an actin depolymerizing factor (ADF) that is implicated in aggressive cancer cell behavior (12-17), was selected for further validation. The main function of cofilin-1 is the depolymerization of F-actin, a biological process crucial for normal mitosis, cytokinesis and cell migration (18). Cofilin-1, was actually up-regulated in all three cancer cell lines (Table I).

Confirmation of the proteomic analysis results. In the 2D gels, cofilin-1 was found up-regulated in HeLa and C-33A cell lines (HeLa/HCK1T ratio: 3.6, C-33A/HCK1T ratio: 3.0). Its levels were also higher in SiHa compared to the ones in HCK1T, but did not reach the 2-fold threshold (SiHa/HCK1T ratio: 1.3) as shown in Figure 6A. The up-regulation of cofilin-1 in the 2D gels was confirmed by western blot analysis on the four cell lines (Figure 6B). As shown in Figure 6, the levels of cofilin-1 in the western blot analysis follow the same expression pattern as in the 2D gels, thus confirming the proteomic analysis results.

Discussion

The comparison of proteomic profiles between the three cervical cancer cell lines (HeLa, SiHa, C-33A) and the normal cervical keratinocytes (HCK1T) total cell extract, revealed a total of 113 differentially expressed proteins (cancer/normal ratio <0.5 or >2). Around 60% of these proteins are associated to cytoskeleton or metabolism. Moreover, bioinformatics analysis of the differentially expressed proteins revealed that actin cytoskeleton signaling represents a statistically significant pathway deregulated in cervical cancer. This finding led us to the investigation of cofilin-1, in the context of cervical cancer.
and actin cytoskeleton remodeling, and the independent confirmation of its expression trend by western blot analysis.

Functional annotation of the differentially expressed proteins indicated that nearly 63% of all deregulated proteins are involved in metabolism. More specifically, 67% of the proteins involved in metabolism are up-regulated in the cervical cancer cell lines compared to normal cervical keratinocytes. This finding could serve as a validation of our approach, since uncontrolled proliferation occurring during carcinogenesis, generates additional anabolic and energy demands. To sustain survival and proliferation, cancer cells have to activate or enhance metabolic pathways that utilize the available nutrients for production of metabolic precursors for cell anabolism, and maintain the reduction–oxidation balance (19). Hence, molecules and regulators that participate in such metabolic processes are expected to be deregulated in cancer cells when compared to normal ones. Such typical examples of enzyme proteins that emerged from our analysis, included alpha-enolase; triosephosphate isomerase; glyceraldehyde-3-phosphate dehydrogenase; peptidyl-prolyl cis-trans isomerase A; fructose-bisphosphate aldolase A; and peroxiredoxins 1, 2, 5 and 6.

Actin cytoskeleton signaling, a statistically significant pathway that emerged from bioinformatics analysis of the differentially expressed proteins, is known to play a pivotal role in cancer, since actin cytoskeleton remodeling is essential for cell proliferation and migration. The process, during which a cancer cell migrates from the original site of the tumor to a new site, consists of specific steps, known as the ‘metastatic cascade’. To metastasize, a cancer cell has to detach from the primary tumor site, migrate, intravasate, translocate through vessels, extravasate, and finally, attach and grow a secondary tumor at a new site. Due to their plasticity, cancer cells can move utilizing either mesenchymal or amoeboid motility, depending on the physical properties of the extracellular matrix, the degree of extracellular proteolysis and on the soluble signaling factors. This whole procedure requires extensive cell cytoskeleton reorganization to achieve the desired cell movements and shape alterations. Actin cytoskeleton remodeling requires the fine orchestration among actin microfilaments, intermediate filaments and microtubules (20, 21).
Table 1. List of differentially expressed proteins following comparison between normal and cancer cervical cell lines.

A. Proteins down-regulated in the cervical cancer cell lines compared to normal cervical keratinocytes (HCK1T).

| Spot # | Uniprot ID     | Protein                                      | Mascot score | Molecular weight (Da) | pI     | Expression fold change | HeLa/ HCK1T | SiHa/ HCK1T | C-33A/ HCK1T |
|--------|----------------|----------------------------------------------|--------------|-----------------------|--------|------------------------|-------------|-------------|--------------|
| 3      | 1433S_HUMAN    | 14-3-3 protein sigma                         | 91           | 27871                 | 4.5    | 0.145                  | 0.246       | 0.082       |
| 6      | VIME_HUMAN     | Vimentin                                     | 161          | 53676                 | 4.9    | 0.461                  | N/A         |
| 7      | HSPB1_HUMAN    | Heat shock protein beta-1                   | 78           | 22826                 | 6.0    | 0.324                  | 0.376       |
| 8      | TERA_HUMAN     | Transitional endoplasmic reticulum ATPase    | 57           | 89950                 | 5.0    | 0.284                  | 0.065       |
| 9      | HSP7C_HUMAN    | Heat shock cognate 71 kDa protein            | 81           | 71082                 | 5.2    | 0.429                  | 0.330       |
| 10     | HSPF1_HUMAN    | Heat shock 70 kDa protein 1A/1B              | 85           | 70294                 | 5.4    |                        |             |
| 11     | HSPB1_HUMAN    | Heat shock protein beta-1                   | 119          | 22826                 | 6.0    | 0.353                  | 0.269       |
| 15     | HSPB1_HUMAN    | Heat shock protein beta-1                   | 85           | 22826                 | 6.0    | 0.134                  | 0.071       | 0.127       |
| 17     | SPB5_HUMAN     | Serpin B5                                    | 65           | 45250                 | 5.7    | 0.356                  | 0.132       | 0.123       |
| 20     | HRNH1_HUMAN    | Heterogeneous nuclear ribonucleoprotein H    | 85           | 49484                 | 5.9    | 0.383                  |             |
| 22     | CAPG_HUMAN     | Macrophage-capping protein                   | 51           | 38760                 | 5.8    | 0.201                  |             |
| 23     | HSPB1_HUMAN    | Heat shock protein beta-1                   | 131          | 22826                 | 6.0    | 0.260                  | 0.381       | 0.023       |
| 24     | K2C6A_HUMAN    | Keratin, type II cytoskeletal 6A             | 49           | 60293                 | 8.9    | 0.176                  | 0.380       |
| 28     | WDR1_HUMAN     | WD repeat-containing protein 1               | 76           | 66836                 | 6.2    | 0.287                  | 0.468       |
| 29     | PUR9_HUMAN     | Bifunctional purine biosynthesis protein PURH| 80           | 65089                 | 6.3    |                        | 0.404       |
| 30     | PDLI1_HUMAN    | PDZ and LIM domain protein 1                | 187          | 36505                 | 6.6    |                        | N/A         |
| 33     | K2C5_HUMAN     | Keratin, type II cytoskeletal 5              | 120          | 62568                 | 8.6    | 0.101                  |             |
| 35     | FSCN1_HUMAN    | Fascin                                       | 103          | 55123                 | 7.0    | 0.452                  | 0.420       |
| 36     | FSCN1_HUMAN    | Fascin                                       | 159          | 55123                 | 7.0    | 0.200                  | 0.091       | 0.058       |
| 37     | K2C5_HUMAN     | Keratin, type II cytoskeletal 5              | 97           | 62568                 | 8.6    | 0.164                  | 0.129       | 0.224       |
| 38     | K2C6A_HUMAN    | Keratin, type II cytoskeletal 6A             | 98           | 60293                 | 8.9    | 0.032                  | 0.019       | 0.044       |
| 39     | K2C6B_HUMAN    | Keratin, type II cytoskeletal 6B             | 88           | 60315                 | 8.9    |                        |             |
| 40     | FSCN1_HUMAN    | Keratin, type II cytoskeletal 6C             | 75           | 60273                 | 8.9    |                        |             |
| 41     | FSCN1_HUMAN    | Fascin                                       | 237          | 55123                 | 7.0    | 0.200                  | 0.091       | 0.058       |
| 42     | K2C5_HUMAN     | Keratin, type II cytoskeletal 5              | 146          | 62568                 | 8.6    | 0.314                  |             |
| 43     | K2C6A_HUMAN    | Keratin, type II cytoskeletal 6A             | 143          | 60293                 | 8.9    |                        |             |
| 44     | K2C6B_HUMAN    | Keratin, type II cytoskeletal 6B             | 124          | 60315                 | 8.9    |                        |             |
| 45     | K2C6C_HUMAN    | Keratin, type II cytoskeletal 6C             | 111          | 60273                 | 8.9    |                        |             |
| 46     | KCRU_HUMAN     | Creatine kinase U-type, mitochondrial        | 60           | 47406                 | 9.4    | 0.324                  | 0.457       |
| 47     | AXA2L_HUMAN    | Putative annexin A2-like protein             | 141          | 38808                 | 8.5    | 0.440                  | 0.044       |
| 48     | CALR_HUMAN     | Calreticulin                                 | 188          | 48283                 | 4.1    |                        | 0.447       |
| 52     | PDIA1_HUMAN    | Protein disulfide-isomerase                  | 143          | 57480                 | 4.6    | 0.140                  |             |
| 55     | GRP78_HUMAN    | 78 kDa glucose-regulated protein             | 105          | 72402                 | 4.9    | 0.305                  |             |
| 60     | EIF3I_HUMAN    | Eukaryotic translation initiation factor 3 subunit I| 113       | 36878                 | 5.3    | 0.499                  |             |
| 62     | XRC5C_HUMAN    | X-ray repair cross-complementing protein 5   | 63           | 83222                 | 5.5    | 0.369                  |             |
| 63     | RLK0_HUMAN     | 60S acidic ribosomal protein P0              | 114          | 34423                 | 5.6    | 0.401                  |             |
| 75     | VINC_HUMAN     | Vinculin                                     | 63           | 124292                | 5.4    | 0.125                  |             |
| 86     | ACTG_HUMAN     | Actin, cytoplasmic 2                         | 53           | 42108                 | 5.2    | 0.336                  |             |
| 89     | IMDH2_HUMAN    | Inosine-5'-monophosphate dehydrogenase 2    | 89           | 56226                 | 6.5    | 0.199                  | 0.284       |
| 90     | FSCN1_HUMAN    | Fascin                                       | 64           | 55123                 | 7.0    |                        |             |
| 100    | FUBP1_HUMAN    | Far upstream element-binding protein 1       | 81           | 67690                 | 7.8    | 0.478                  |             |
| 120    | HSP71_HUMAN    | Heat shock 70 kDa protein 1A/1B              | 159          | 70294                 | 5.4    | 0.369                  |             |
| 123    | TB5B_HUMAN     | Tubulin beta chain                           | 122          | 50095                 | 4.6    | 0.114                  |             |
| 124    | TBB2C_HUMAN    | Tubulin beta-2C chain                        | 111          | 50255                 | 4.7    | 0.059                  |             |
| 125    | ATPB_HUMAN     | ATP synthase subunit beta, mitochondrial     | 106          | 56525                 | 5.1    | 0.124                  |             |
| 126    | TBB4_HUMAN     | Tubulin beta-4 chain                         | 101          | 50010                 | 4.6    |                        |             |
Table I. Continued

| Spot # | Uniprot ID | Protein                                      | Mascot score | Molecular weight (Da) | pI | Expression fold change |
|--------|------------|----------------------------------------------|--------------|-----------------------|----|------------------------|
|        |            |                                              |              |                       |    | HeLa/ HCK1T  | SiHa/ HCK1T | C-33A/ HCK1T |
| 127    | TBB2A_HUMAN | Tubulin beta-2A chain                        | 101          | 50274                 | 4.6| 2,480                  |             |
| 162    | K2C8_HUMAN  | Keratin, type II cytoskeletal 8              | 81           | 53671                 | 5.4| 0.159                  | N/A         |
| 174    | PGK1_HUMAN  | Phosphoglycerate kinase 1                    | 149          | 44985                 | 9.2| 0.419                  |             |
| 176    | TAGL2_HUMAN | Transgelin-2                                 | 144          | 22548                 | 9.3| 0.255                  | N/A         |
| 179    | ATPB_HUMAN  | ATP synthase subunit beta, mitochondrial     | 167          | 56525                 | 5.1| 0.465                  |             |
|        | TBB5_HUMAN  | Tubulin beta chain                           | 64           | 50095                 | 4.6| 13,780                 | 2,460       |
|        | TBB2B_HUMAN | Tubulin beta-2B chain                        | 58           | 50377                 | 4.6| 6,530                  |             |
|        | TBB2A_HUMAN | Tubulin beta-2A chain                        | 58           | 50274                 | 4.6| 6,530                  |             |
| 207    | ATPA_HUMAN  | ATP synthase subunit alpha, mitochondrial    | 107          | 59828                 | 9.6| 0.344                  | 0.412       |
|        | GLYM_HUMAN  | Serine hydroxymethyltransferase, mitochondrial| 70          | 56414                 | 9.5|                       |             |

B. Proteins up-regulated in the cancer cervical cell lines compared to normal cervical keratinocytes (HCK1T).

| Spot # | Uniprot ID | Protein                                      | Mascot score | Molecular weight (Da) | pI | Expression fold change |
|--------|------------|----------------------------------------------|--------------|-----------------------|----|------------------------|
|        |            |                                              |              |                       |    | HeLa/ HCK1T  | SiHa/ HCK1T | C-33A/ HCK1T |
| 53     | RSSA_HUMAN  | 40S ribosomal protein SA                      | 55           | 32947                 | 4.6| 2,480                  |             |
| 54     | EF1D_HUMAN  | Elongation factor 1-alpha                    | 51           | 31217                 | 4.8| 11,990                 |             |
| 57     | CH60_HUMAN  | 60 kDa heat shock protein, mitochondrial      | 187          | 61187                 | 5.6| 2,430                  | 2,380       |
| 59     | TCPE_HUMAN  | T-complex protein 1 subunit epsilon          | 73           | 60089                 | 5.3| 4,020                  |             |
| 61     | IPYR_HUMAN  | Inorganic pyrophosphatase                    | 109          | 33095                 | 5.5| 2,090                  |             |
| 65     | PDI3A3_HUMAN| Protein disulfide-isomerase A3               | 200          | 57146                 | 5.9| 2,450                  |             |
| 66     | LDHB_HUMAN  | L-lactate dehydrogenase B chain              | 64           | 36900                 | 5.7| 2,280                  |             |
| 68     | NDKA_HUMAN  | Nucleoside diphosphate kinase A              | 107          | 17309                 | 5.8| 2,520                  |             |
| 69     | TCPA_HUMAN  | T-complex protein 1 subunit alpha            | 126          | 60819                 | 5.7| 3,580                  | 3,190       |
| 70     | SBP1_HUMAN  | Selulin-binding protein 1                    | 152          | 52928                 | 5.9| 3,110                  |             |
| 71     | ENOA_HUMAN  | Alpha-enolase                                | 78           | 47481                 | 7.7| 2,150                  | 2,690       |
| 73     | TPI5_HUMAN  | Triosephosphate isomerase                    | 113          | 31057                 | 5.6| 3,210                  | 3,990       |
| 74     | LMNA_HUMAN  | Prelamin-A/C                                 | 112          | 74380                 | 6.6| 6,780                  |             |
| 76     | ERP29_HUMAN | Endoplasmic reticulum resident protein 29    | 121          | 29032                 | 7.5| 10,200                 | 8,170       |
| 77     | FABP5_HUMAN | Fatty acid-binding protein, epidermal        | 78           | 15497                 | 7.5| 6,340                  |             |
| 78     | LMNA_HUMAN  | Prelamin-A/C                                 | 101          | 74380                 | 6.6| 3,480                  |             |
| 79     | SERA_HUMAN  | D-3-phosphoglycerate dehydrogenase           | 70           | 57356                 | 6.3| 10,630                 | 2,770       |
| 80     | EFIG_HUMAN  | Elongation factor 1-gamma                    | 138          | 50429                 | 6.3| 7,570                  | 5,570       |
| 81     | HINT2_HUMAN | Histidine triad nucleotide-binding protein 2 | 50           | 17208                 | 9.8| 3,900                  |             |
| 82     | TCPZ_HUMAN  | T-complex protein 1 subunit zeta             | 66           | 58444                 | 6.2| 5,890                  | 2,920       |
|        | STIP1_HUMAN | Stress-induced-phosphoprotein 1              | 62           | 63227                 | 6.4| 2,120                  |             |
|        | LMNA_HUMAN  | Prelamin-A/C                                 | 60           | 74380                 | 6.6| 2,120                  |             |
|        | ENOA_HUMAN  | Alpha-enolase                                | 116          | 47481                 | 7.7| 4,600                  | 2,430       |
|        | STIP1_HUMAN | Stress-induced-phosphoprotein 1              | 93           | 63227                 | 6.4| 3,390                  |             |
|        | SERA_HUMAN  | D-3-phosphoglycerate dehydrogenase           | 149          | 57356                 | 6.3| 3,350                  |             |
|        | PGAM1_HUMAN | Phosphoglycerate mutase                      | 144          | 28900                 | 6.8| 2,710                  | 3,270       |
| 96     | GUAA_HUMAN  | GMP synthase [glutamine-hydrolyzing]         | 120          | 77408                 | 9.8| 3,900                  |             |
| 97     | EF2_HUMAN   | Elongation factor 2                          | 60           | 96246                 | 6.4| 2,320                  |             |
| 98     | EF2_HUMAN   | Elongation factor 2                          | 60           | 96246                 | 6.4| 2,320                  |             |
|        | PS2A_HUMAN  | Proteasome subunit alpha type-2             | 95           | 25996                 | 7.7| 2,040                  |             |
| 103    | EF2_HUMAN   | Elongation factor 2                          | 72           | 96246                 | 6.4| 3,050                  |             |
| 104    | K2C1_HUMAN  | Keratin, type II cytoskeletal 1              | 47           | 66170                 | 8.8| 13,780                 | 2,460       |
| 107    | GBLP_HUMAN  | Guanine nucleotide-binding protein subunit beta-2-like 1 | 177          | 35511                 | 8.9| 4,760                  |             |
| 108    | RAN_HUMAN   | GTP-binding nuclear protein Ran              | 133          | 24579                 | 7.8| 3,550                  | 2,600       |
| 109    | HCD2_HUMAN  | 3-hydroxyacyl-CoA dehydrogenase type-2       | 145          | 27134                 | 9.1| 8,720                  | 5,035       |
| 110    | PPIA_HUMAN  | Peptidyl-prolyl cis-trans isomerase A        | 91           | 18229                 | 9.0| 3,060                  |             |
| 112    | PRDX5_HUMAN | Peroxiredoxin-5, mitochondrial               | 81           | 22301                 | 9.9|                       |             |
| Spot # | Uniprot ID | Protein                                      | Mascot score | Molecular weight (Da) | pI | Expression fold change |
|--------|------------|----------------------------------------------|--------------|-----------------------|----|-----------------------|
|        |            |                                              | HeLa/         | SiHa/                 | C-33A/ |                     |
|        |            |                                              | HCK1T         | HCK1T                 | HCK1T |                     |
| 111    | PPIA_HUMAN | Peptidyl-prolyl cis-trans isomerase A         | 113          | 18229                 | 9.0 | 2.610                |
| 112    | KPYM_HUMAN | Pyruvate kinase isozymes M1/M2               | 258          | 58470                 | 9.0 | 4.620                |
| 113    | ALDOA_HUMAN| Fruktose-bisphosphate aldolase              | 140          | 39851                 | 9.2 | 2.520                |
| 114    | LDHA_HUMAN | L-lactate dehydrogenase A chain              | 92           | 36950                 | 9.3 | 3.940                |
|        |            |                                              | 17,930       |                       |     |                       |
| 115    | PRDX1_HUMAN| Peroxiredoxin-1                              | 152          | 22234                 | 9.2 | 13.960               |
| 116    | PPIA_HUMAN | Peptidyl-prolyl cis-trans isomerase A         | 84           | 18229                 | 9.0 | 3.660                |
| 117    | COF1_HUMAN | Cofilin-1                                    | 99           | 18719                 | 9.1 | 4.670                |
| 118    | G3P_HUMAN  | Glyceraldehyde-3-phosphate dehydrogenase     | 52           | 36201                 | 9.3 | 2.440                |
| 125    | K2C7_HUMAN | Keratin, type II cytoskeletal 7              | 61.00        | 21411                 | 5.3 | 3.350                |
|        |            |                                              | 3,730        |                       |     |                       |
| 126    | APT_HUMAN  | Adenine phosphoribosyltransferase            | 94           | 19767                 | 5.7 | 7.510                |
| 128    | RFA2_HUMAN | Replication protein A 32 kDa subunit         | 57           | 29342                 | 5.7 | 2.380                |
| 139    | ERP29_HUMAN| Endoplasmic reticulum resident protein 29    | 61           | 29032                 | 7.5 | 2.030                |
| 143    | A26L1_HUMAN| Putative ankyrin repeat domain-containing     | 47           | 14164                 | 9.6 | 3.460                |
|        |            | protein 26-like 1                            |              |                       |     | 14,730               |
| 144    | PARK7_HUMAN| Protein DJ-1                                 | 93           | 20050                 | 6.4 | 2.400                |
| 145    | TCPB_HUMAN | T-complex protein 1 subunit beta             | 112          | 57794                 | 6.0 | 2.700                |
| 148    | LSHB_HUMAN | Lutropin subunit beta                        | 51           | 16019                 | 9.9 | 2.040                |
| 152    | SCOT1_HUMAN| Succinyl-CoA:3-ketocoezyme A transferase 1, mitochondrial| 70 | 56578 | 7.8 | 5.990 | 10,220 |
| 158    | KIC10_HUMAN| Keratin, type I cytoskeletal 10              | 56           | 59020                 | 5.0 | 4.370                |
| 160    | PNP1_HUMAN | Purine nucleoside phosphorylase              | 82           | 30325                 | 6.5 | 3.890                |
| 161    | PS2A_HUMAN | Proteasome subunit alpha type-2              | 67           | 25996                 | 7.7 | 10.690               |
| 163    | ALDR_HUMAN | Aldose reductase                             | 139          | 36230                 | 6.6 | 5.050                |
| 165    | LSHB_HUMAN | Lutropin subunit beta                        | 47           | 16019                 | 9.9 | 2.140                |
| 167    | IDHC_HUMAN | Isocitrate dehydrogenase [NADP] cytoplasmic | 199          | 46915                 | 6.6 | 11.680               |
| 168    | ALDR_HUMAN | Aldose reductase                             | 172          | 36230                 | 6.6 | 3.730                |
| 169    | PPIA_HUMAN | Peptidyl-prolyl cis-trans isomerase A         | 47           | 18229                 | 9.0 | 2.860                |
| 170    | LSHB_HUMAN | Lutropin subunit beta                        | 48           | 16019                 | 9.9 | 2.920                |
| 172    | ALDR_HUMAN | Aldose reductase                             | 80           | 36230                 | 6.6 | 3.260                |
| 173    | PERP1_HUMAN| Phosphatidylethanolamine-binding protein 1    | 82           | 21411                 | 5.3 | 3.140                |
| 175    | LEG3_HUMAN | Gaelectin-3                                   | 66           | 26193                 | 9.1 | 2.270                |
| 181    | ATP5H_HUMAN| ATP synthase subunit d, mitochondrial         | 56           | 18537                 | 5.1 | 5.310                |
| 183    | PHB_HUMAN  | Prohibitin                                   | 98           | 29843                 | 5.5 | 11.430               |
| 184    | PRDX2_HUMAN| Peroxiredoxin-2                              | 78           | 22049                 | 5.6 | 4.940                |
| 187    | CH60_HUMAN | 60 kDa heat shock protein, mitochondrial      | 160          | 61187                 | 5.6 | 5.790                |
| 190    | GRB2_HUMAN | Growth factor receptor-bound protein 2        | 80           | 25304                 | 5.9 | 2.290                |
| 191    | PRDX3_HUMAN| Thioredoxin-dependent peroxide reductase, mitochondrial | 57 | 28017 | 8.9 | 5.350 |
|        |            |                                              | 7,380        |                       |     |                       |
| 193    | AL7A1_HUMAN| Alpha-aminoacidic semialdehyde dehydrogenase | 56           | 59020                 | 9.1 | 2.280                |
| 194    | RAN_HUMAN  | GTP-binding nuclear protein Ran               | 65           | 24579                 | 7.8 | 6.210                |
| 196    | TPIS_HUMAN | Triosephosphate isomerase                    | 84           | 31057                 | 5.6 | 2.050                |
| 197    | AL7A1_HUMAN| Alpha-aminoacidic semialdehyde dehydrogenase | 71           | 59020                 | 9.1 | 7.110                |
| 198    | EFTU_HUMAN | Elongation factor Tu, mitochondrial           | 202          | 24952                 | 7.9 | 2.060                |
| 199    | PS2B_HUMAN | Proteasome subunit beta type-2               | 61           | 22993                 | 6.6 | 5.560                |
| 200    | EFTA_HUMAN | Electron transfer flavoprotein subunit alpha, mitochondrial | 51 | 35400 | 9.5 | 2.840 |
|        |            |                                              | 3,830        |                       |     |                       |
| 201    | GBLP_HUMAN | Guanine nucleotide-binding protein subunit beta-2-like 1 | 138 | 35511 | 8.9 | 3.160 |
|        |            |                                              | 12,500       |                       |     |                       |
| 203    | COF1_HUMAN | Cofilin-1                                    | 55           | 18719                 | 9.1 | 2.430                |
| 205    | VDAC2_HUMAN| Voltage-dependent anion-selective channel protein 2 | 94 | 32060 | 8.7 | 6.060 |
| 206    | PSA4_HUMAN | Proteasome subunit alpha type-4              | 63           | 29750                 | 8.7 | 2.400                |
| 208    | VDAC1_HUMAN| Voltage-dependent anion-selective channel protein 1 | 89 | 30868 | 9.2 | 3.730 |
| 209    | ETFB_HUMAN | Electron transfer flavoprotein subunit       | 55           | 28054                 | 9.2 | 3.120                |
| 210    | PRDX6_HUMAN| Peroxiredoxin-6                              | 88           | 25133                 | 6.0 | 11,580               |
| 211    | VDAC1_HUMAN| Voltage-dependent anion-selective channel protein 1 | 82 | 30868 | 9.2 | 17,930 |
| 212    | PSA7_HUMAN | Proteasome subunit alpha type-7              | 75           | 28041                 | 9.3 | 2.340                |

Table I. Continued
In our study, cofilin-1, which is involved in the Actin cytoskeleton signaling, was actually up-regulated in HeLa and C-33A (>2-fold expression change), while it was also found in higher levels in SiHa (1.3-fold expression change) compared to HCK1T. This expression trend was confirmed by western blot analysis on the four cell lines total cell extract, employing an antibody previously validated and recommended for use in western blot, according to the guidelines (22).

Cofilin-1 is a small protein of ~19 kD whose name stands for cofilamentous protein. It plays a key role in actin dynamics, cell division, chemotaxis and cancer cell migration (18, 23). Cofilin-1 severs and depolymerizes actin filaments, thus increasing the free barbed ends where actin polymerization occurs (24, 25). However, different concentrations of cofilin-1 have different effects on actin filament severing and nucleation. Low concentration favors severing, while high concentration favors nucleation (26). Cofilin-1 also has a Nuclear Localization Signal (NLS) and can transfer monomeric actin in the nucleus where rod-like structures of actin are formed in response to heat shock, ATP-depletion and dimethyl sulfoxide (DMSO) treatment, cytochalasin D or high cytosolic G-actin concentration. However, its biological role in the nucleus remains unclear (18).
Figure 4. Ingenuity® Canonical Pathways deregulated between normal and cancer cervical cell lines. Only statistically significant pathways are shown in a descending order of p-value using Fisher’s exact test.

Table III. List of Ingenuity® Pathway Analysis results from the comparison between normal and cancer cervical cell lines. Only statistically significant pathways are shown in a descending order of p-value, using Fisher’s exact test.

| Ingenuity® Canonical Pathways                                                                 | p-Value     | Differentially expressed proteins from our dataset                                                                 |
|------------------------------------------------------------------------------------------------|-------------|------------------------------------------------------------------------------------------------------------------|
| Unfolded protein response                                                                      | 4.17E-10    | CALR, HSPA1A/HSPA1B, HSPA9, HSPA8, HSPA5, VCP, HSPA6, P4HB                                                        |
| Remodeling of Epithelial Adherens Junctions                                                    | 2.82E-09    | TUBB2A, TUBB4A, VCL, ACTG1, TUBB, TUBB2B, NME1, TUBB4B                                                             |
| Protein Ubiquitination Pathway                                                                | 1.17E-07    | HSPB1, HSPA1A/HSPA1B, HSPA9, PSMA4, PSMA7, HSPA8, HSPA5, HSPD1, PSMA2, PSMB2, HSPA6                             |
| Glycolysis I                                                                                   | 1.91E-07    | TPI1, PGAM1, PGK1, GAPDH, ALDOA                                                                                   |
| 14-3-3-mediated Signaling                                                                     | 2.09E-07    | TUBB2A, VIM, TUBB4A, SFN, TUBB, TUBB2B, GRB2, TUBB4B                                                             |
| Mitochondrial Dysfunction                                                                      | 3.31E-07    | HSD17B10, VDAC1, PARK7, PRDX3, VDAC2, ATP5A1, ATP5H, PRDX3, ATP5B                                                 |
| Glucocorticoid Signaling                                                                        | 8.91E-06    | PGAM1, PGK1, GAPDH, ALDOA                                                                                         |
| Aldosterone Signaling in Epithelial Cells                                                      | 1.70E-05    | HSPB1, HSPA1A/HSPA1B, HSPA9, HSPA8, HSPA5, HSPD1, HSPA6                                                          |
| Gap Junction Signaling                                                                         | 1.95E-05    | TUBB2A, TUBB4A, ACTG1, TUBB, TUBB2B, GRB2, TUBB4B                                                                |
| Purine Nucleotides De Novo Biosynthesis II                                                    | 3.09E-05    | IMPDH2, GMPS, ATIC                                                                                               |
| Breast Cancer Regulation by Stathmin1                                                          | 7.41E-05    | TUBB2A, TUBB4A, NGN2, TNFR1, TUBB, TUBB2B, GRB2, TUBB4B                                                           |
| eNOS Signaling                                                                                 | 9.55E-04    | HSPA1A/HSPA1B, HSPA9, HSPA8, HSPA5, HSPA6, GRB2, TUBB4B                                                          |
| VEGF Signaling                                                                                 | 1.41E-03    | SFN, VCL, ACTG1, GRB2                                                                                             |
| Glucocorticoid Receptor Signaling                                                              | 2.75E-03    | HSPA1A/HSPA1B, HSPA9, HSPA8, HSPA5, GRB2, HSPA6                                                                |
| NRF2-mediated Oxidative Stress Response                                                        | 2.75E-03    | ACTG1, ERP29, VCP, PRDX1, STIP1                                                                                   |
| FAK Signaling                                                                                  | 1.12E-02    | VCL, ACTG1, GRB2                                                                                                 |
| Death Receptor Signaling                                                                        | 1.32E-02    | HSPB1, ACTG1, LMNA                                                                                                |
| EIF2 Signaling                                                                                 | 1.74E-02    | GRB2, EIF3I, RPLP0, RPSA                                                                                          |
| Paxillin Signaling                                                                             | 1.74E-02    | VCL, ACTG1, GRB2                                                                                                |
| Oxidative Phosphorylation                                                                      | 2.04E-02    | ATP5A1, ATP5H, ATP5B                                                                                              |
| Actin Cytoskeleton Signaling                                                                    | 2.88E-02    | CFL1, VCL, ACTG1, GRB2                                                                                            |
| Signaling by Rho Family GTPases                                                                | 3.63E-02    | VIM, CFL1, ACTG1, GRB2                                                                                            |
| Regulation of eIF4 and p70S6K Signaling                                                        | 4.37E-02    | GRB2, EIF3I, RPSA                                                                                                |
Figure 5. Actin cytoskeleton signaling is a statistically significant affected pathway in cervical cancer cells, as documented from the Ingenuity® Pathway Analysis of the differentially expressed proteins, between cervical cancer cell lines and normal cervical keratinocytes. Nodes filled with red color represent proteins that were found up-regulated in cervical cancer cell lines and nodes filled with green color represent proteins that were found down-regulated in the cervical cancer cell lines. Double-lined nodes represent groups of proteins or complexes.

Figure 6. Cofilin-1 levels in cervical cell lines. A. Cofilin-1 levels in proteomics analysis. Fold change is expressed in comparison with HCK1T, as the average from the two identified spots on 2D gels, measured with PDQuest software. B. Cofilin-1 levels in western blot analysis. Fold change is expressed in comparison with HCK1T representing the average from four biological replicates for each cell line, measured with the Quantity One 1-D Analysis software (Bio-Rad). Tubulin levels were used for signal normalization in western blots. Mean values and standard deviation bars are shown for each cell line, and p-value was calculated with Student’s t-test.
The function of cofilin-1 does not only depend on its concentration, but also on several environmental factors. Phosphorylated cofilin-1 on ser-3 is considered to be inactive because of its lower affinity for actin. LIM kinase 1 (LIMK1), LIM kinase 2 (LIMK2) and testicular protein kinase 1/2 (TESK1/2) phosphorylate cofilin-1 on ser3, while slingshot-1L (SSH1L) phosphatase and chronophin (CIN) induce its dephosphorylation (18). Upon stimulation by epidermal growth factor (EGF), cofilin-1 becomes activated through dephosphorylation and dissociation from phosphatidylinositol 4,5-bisphosphate (PIP2) in order to reorganize the cytoskeleton for chemiotactic migration (27). Changes on the intracellular pH, regulated by Na\(^+\)-H\(^+\) exchanger 1 (NHE1), can also affect cofilin-1 activity. When pH is higher than normal (6.8-7.4), cofilin-1 can be also activated through dissociation from the inhibitory complexes with cortactin and PIP2 (25, 28, 29).

In the context of cancer metastasis and cell migration, cofilin-1 is active at the leading edge of migrating cells protrusions and its levels have been studied in various types of cancer (25). In non-small cell lung cancer (NSCLC), high cofilin-1 levels were correlated with lower overall survival rate, cellular invasiveness and resistance to drugs, and particularly, to cisplatin (30-33). Coflin-1 was also found up-regulated in breast cancer and its levels correlated with tumor size and stage (34-36). Overexpression of cofilin-1 can predict shorter progression-free survival in advanced ovarian cancer patients, receiving standard therapy (37). Immunohistochemistry studies on prostate tissue sections revealed expression of cofilin-1 in 70% of prostate cancer samples, while benign prostate hyperplasia samples were negative for the protein. In the same study, coflin-1 levels were significantly associated with the Gleason score and the presence of lymph node metastasis (38). Furthermore, proteomic analysis of saliva from patients with head and neck squamous cell carcinoma, revealed significantly increased levels of cofilin-1 compared to the control group (39). Additionally, high expression levels of cofilin-1 were associated with large tumor size, high TNM stage, lymph node metastasis, and decreased overall survival in immunohistochemical studies of patients with squamous cell and adenosquamous carcinoma, and adenocarcinoma of the gallbladder (40). Finally, high levels of cofilin-1 expression compared to normal tissue have also been documented in pancreatic cancer (41) and oral carcinoma (42).

As mentioned above, in our study, the differentially expressed proteins were obtained from the comparison of normal cervical keratinocytes with the three different cervical cancer cell lines. These cervical cancer cell lines differ primarily by the presence or absence and the type of the HPV. Specifically, HeLa is positive for HPV18, SiHa is positive for HPV16, and C-33A is negative for HPV. These cell lines were carefully chosen for our analysis in order to investigate the differences between the HPV-positive and HPV-negative types of cervical cancers. The availability of the 18 informative differentially expressed proteins (Figure 3 and Table II) revealed from these two-way comparisons among the four cell lines, provide the impetus for further functional studies to dissect the molecular mechanisms that could play a role in the two distinct pathways of cervical carcinogenesis.

Our study is the first to provide an insight into the differences of the total proteome of cervical cancer cell lines in direct comparison to normal cervical keratinocytes. Most of the proteomic studies utilizing cervical cancer cell lines, focus on the differences that occur in the protein expression pattern as an effect of a drug treatment, such as doxorubicin, oxymatrine and cisplatin (43-45), or under stress conditions, like UVB irradiation and hypoxia (46, 47), or after the induced and/or inhibited expression of specific genes, as in the cases of HVP16 E6 gene, transgelin-2 and parkin (48-50). Moreover, our group has recently studied the secretome of these cervical cancer cell lines compared to normal cervical keratinocytes (8). Comparative analysis of the secretome revealed 67 differentially expressed proteins, out of which 36 were also identified as differentially expressed in our study. Furthermore, Lin et al. (51) proposed several putative biomarkers for the rare and very aggressive type of neuroendocrine cervical cancer by comparing the proteomic profile of HM-1, a neuroendocrine cervical cancer cell line, to CaSki, ME-180, and HeLa, that exhibit a non-neuroendocrine origin (51). Their study disclosed 82 differentially expressed proteins and further confirmed the differential expression of transgelin, galectin-1 and PGK-1 in all cell lines employing western blotting. Interestingly, transgelin and PGK-1 were also found differentially expressed in our analysis, suggesting a pivotal role of these proteins in cervical pathology.

In conclusion, the proteomic comparison of the three cervical cancer cell lines with normal cervical keratinocytes, revealed novel proteins that are potentially deregulated in cervical cancer and could be further investigated as putative biomarkers and pharmacological targets. Moreover, bioinformatics analysis indicated that these proteins are involved in processes and pathways that are already documented to be active during carcinogenesis, confirming the validity of the proteomics results. The expression trend of cofilin-1, that was found up-regulated in the cancer group, was confirmed by western blot. Although cofilin-1 has been studied thoroughly in the context of various types of cancer, to our knowledge, it has not been studied yet in cervical cancer. Therefore, the up-regulation of the protein in the cancer cell lines we documented, indicates that cofilin-1 could also be overexpressed in cervical cancer biopsies. Nevertheless, confirmation of this hypothesis in a cohort of well-characterized clinical samples of different clinical stages is definitely needed, and could lead to the use of...
cofilin-1 as a valuable marker of either cancerous and/or precancerous lesions of the cervical epithelium.

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