Quantitative Proteomic Analysis of Cervical Cancer Tissues Identifies Proteins Associated With Cancer Progression

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Abstract. *Background/Aim:* To date, several proteomics studies in cervical cancer (CC) have focused mainly on squamous cervical cancer (SCC). Our study aimed to discover and clarify differences in SCC and CAD that may provide valuable information for the identification of proteins involved in tumor progression, in CC as a whole, or specific for SCC or CAD. *Materials and Methods:* Total protein extracts from 15 individual samples corresponding to 5 different CC tissue types were compared with a non-cancerous control group using bidimensional liquid chromatography-mass spectrometry (2D LC-MS/MS), isobaric tags for relative and absolute quantitation (iTRAQ), principal component analysis (PCA) and gene set enrichment analysis (GSEA). *Results:* A total of 622 statistically significant different proteins were detected. Exocytosis-related proteins were the most over-represented, accounting for 25% of the identified and quantified proteins. Based on the experimental results, reticulocalbin 3 (RCN3) and Ras-related protein Rab-14 (RAB14) were chosen for further downstream in vitro and vivo analyses. RCN3 was overexpressed in all CC tissues compared to the control and RAB14 was overexpressed in squamous cervical cancer (SCC) compared to invasive cervical adenocarcinoma (CAD). In the tumor xenograft experiment, RAB14 protein expression was positively correlated with increased tumor size. In addition,

RCN3-expressing HeLa cells induced a discrete size increment compared to control, at day 47 after inoculation. *Conclusion:* RAB14 and RCN3 are suggested as potential biomarkers and therapeutic targets in the treatment of CC.

Cervical cancer (CC) is the fourth most frequently diagnosed cancer in women, with an estimated 265,000 deaths worldwide (1). A large majority (around 80%) of the global burden occurs in the less developed countries (2). Careful retrospective investigations have strongly implicated high-risk human papillomavirus (HPV) with cervical carcinogenesis (3). Although CC diagnosis and treatment programs have decreased the number of cases and mortality rates, the prognosis and complete response to treatment remain poor in CC variants (4, 5). Approximately 80% of the CC cases worldwide are of the squamous cervical cancer (SCC) type, with the remainder being invasive cervical adenocarcinoma (CAD) (6). The incidence of CAD has increased, especially in younger women (7). During the last two decades, the relationship between CAD and SCC and all CCs has doubled even in developed countries. This epidemiological evidence suggests that current screening practices are inadequate to identify a significant proportion of CAD precursor lesions (8).

In a population-based study in Korean women, it was shown that the prevalence of HPV in CAD tissue is approximately 90% (9). The infection of HPV 16 and/or HPV 18 accounted for 78% of HPV-positive CADs, and multiple HPV types were found in the remaining 13% of cases (9). Risk factors for developing CC that have been described in SCC include the use of oral contraceptives for more than five years, smoking, and HPV infection (10). However, nowadays HPV infection is fully accepted as the common trigger factor for SCC and CAD development (11). Considering the high morbidity of the disease and the poor

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prognosis of CC types, it is essential to identify proteins involved in CC development.

Isobaric tags for relative and absolute quantitation (ITRAQ) combined with two-dimensional liquid chromatography (2D LC-MS/MS), are among the most powerful methodologies in quantitative proteomics. The multiplexing scope offered by ITRAQ reagents allowed us to identify and quantify changes in protein expression in CC tissues (SCC and CAD), enabling comparison between them, and of each of them against normal cervix tissue (NCT) used as control. Consequently, proteomics is a useful tool to classify cells and tissues from pathological conditions and understand different biological mechanisms (12).

In this study we used a mass spectrometry-based quantitative proteomics approach to obtain proteomic profiles of SCC and CAD to achieve an integrated analysis by principal component analysis (PCA) and gene set enrichment analysis (GSEA) of differentially expressed proteins. Our analysis revealed several proteins up-regulated in both conditions in comparison to the control. Reticulocalbin 3 (RCN3) and Ras-related protein Rab-14 (RAB14) were selected for further analysis. HeLa cells overexpressing RAB14 and RCN3 separately were inoculated in nu/nu mice to elucidate RCN3 and RAB14 roles in CC. Our data indicated the fundamental role of these proteins in the regulation and progression of CC cells.

Materials and Methods

Samples and reagents. We analyzed 15 CC tissue samples from patients (mean age=45.5 years, SD=12.43) undergoing curative surgery at the Department of Gynecology of the Instituto Nacional de Cancerología (INCan), Mexico City, Mexico. Normal cervix tissues (NCTs) with no evidence of CC and HPV infection were collected from patients attending the Gynecology Department of INCan, for treatment of cervix benign diseases. All the samples were collected with the consent of the patients and the protocol was approved by the Human Ethics Committee of the National Cancer Institute (INCan). After excision, the tissues were flash-frozen in liquid nitrogen and stored at -80°C until later use. A representative part of the tumor area was collected in 10% formalin and embedded in paraffin for histopathological analysis. The clinical and pathological data were recorded on a predesigned form, which included the clinical stage of the tumor, histopathological classification, histopathological differentiation degree, HPV type, and age of the patient. HPV detection was performed by PCR analysis by extracting total DNA from 200 mg of CC tissue samples. DNA isolation was performed using a Wizard Genomic DNA purification Kit (A1120, Promega, Madison, WI, USA). DNA concentration was determined using a NanoDrop 2000/c spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) and visualized on agarose gel. One hundred μg of total DNA from each CC tumor sample was used as a template to detect HPV by PCR using MY09-MY11 HPV primers, designed to amplify a 450-base pair (bp) PCR product (PCRp) from DNA flanking the L1 HPV gene. Amplification was verified by running 10 μl of PCRp sample (out of a total PCR reaction volume of 20 μl) on a 1% agarose gel and staining with ethidium bromide (0.5 $\mu\text{g}/\text{ml}$). A

second set of primers targeting the HPV 18-LCR (618-bp) and HPV 16-E6 (544-bp) regions was used for detecting the two most prevalent high-risk HPV types (HPV 16 and HPV 18), and HPV31 was detected using MY09-MY11 (11). PCRps were isolated with the GeneJET purification kit (K0701, Thermo Fisher Scientific) according to manufacturer's protocol and sequenced by capillary electrophoresis. The DNA sequences were analyzed by the Basic Local Alignment Search Tool (BLAST) at National Center for Biotechnology Information (NCBI). The β -globin housekeeping gene (540 bp) served as a PCR internal control.

The histological diagnosis of each sample was reconfirmed by microscopic examination of a frozen section stained with hematoxylin and eosin (H&E). Two different pathologists performed a histopathological classification according to the Bethesda System (TBS) to confirm cervical vaginal cytologic diagnoses (13). Next, a representative histological section of the tumor was washed 3 times in 1 ml of cold phosphate buffer saline (PBS), pH 7.5, supplemented with protease inhibitors. Frozen samples were crushed and finely homogenized using a handheld homogenizer. Each sample was dissolved in 2 volumes of lysis buffer containing 2 M guanidinium chloride (GdmCl), DNase/RNase, and protease inhibitors for 30 min at 4°C . After sonicating three times for 10 s, the samples were centrifuged at $18,000 \times g$ for 5 min at 4°C . The supernatant was taken for quantitative proteomics analysis, and the protein concentration was determined by the Bradford method (14). Also, each of 6 groups consisted of 3 individual samples, and all 18 samples were analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) to confirm sample integrity (data not shown).

Protein digestion and ITRAQ labeling. Protein digestion and ITRAQ labeling were performed as follows. Six pools, each containing 100 μg of protein from three different tumor protein samples, were precipitated with 9 volumes of cold ethanol at -20°C overnight. The pellet was washed 3 times with cold 90% ethanol. The precipitated proteins were solubilized in 0.5% sodium dodecyl sulfate (SDS) buffer, 0.1 M triethylammonium bicarbonate (TEAB), pH 8.0. For protein digestion, trypsin was added in a 1:50 ratio (enzyme-substrate), and the samples were incubated for 16 h at 37°C . SDS was removed by ethyl acetate extraction under acidic conditions (1 volume of ethyl acetate was added to the sample and acidified with 0.5% trifluoroacetic acid. After vortexing and centrifugation, the organic phase was discarded and an additional ethyl acetate extraction was carried out. Finally, the peptide mixture was desalted by reverse-phase chromatography, dried, and stored at -70°C until ITRAQ labeling. ITRAQ experiments were performed on 6 sets of 3 samples each. A pool of non-paired non-cancerous cervix tissue homogenates was used as a control in each set of experiments: an equal amount of total protein from lysates of 3 non-cancerous samples (non-paired controls) was pooled to generate a common reference "control sample" for CC samples comparison. The samples were dissolved in 40 μl TEAB pH 8.5 each, and the peptide was labeled using the iTRAQ reagent according to the manufacturer's instructions (Applied Biosystems) as follows: HPV16+ SCC poorly differentiated (SqPD16) reagent 114, HPV16+ SCC moderately differentiated (SqMD16) reagent 115, HPV16+ CAD moderately differentiated (AdMD16) reagent 116, HPV18+ SCC moderately differentiated (SqMD18) reagent 117, HPV31+ SCC moderately differentiated (SqMD31) reagent 118 and non-cancerous HPV-negative cervix tissue used as control reagent 121. All reactions were carried out according to the manufacturer's instructions. The peptide-

Figure 1. Schematic view of the experimental design. Proteomics was employed for the discovery of differentially regulated proteins in cervical cancer (CC) tissues and control tissues. The clinical samples were categorized into five sub-groups: human papillomavirus-16-positive (HPV16+) squamous cell cancer (SCC) poorly differentiated (SqPD16), HPV16+ SCC moderately differentiated (SqMD16), HPV16+ cervical adenocarcinoma (CAD) moderately differentiated (AdMD16), HPV18+ SCC moderately differentiated (SqMD18), HPV31+ SCC moderately differentiated (SqMD31) and a control group. Isobaric tags for relative and absolute quantitation (ITRAQ) combined with two-dimensional liquid chromatography (2D LC-MS/MS), are among the most powerful methodologies in quantitative proteomics were used. Principal component analysis (PCA) was performed to obtain through Gene Set Enrichment Analysis (GSEA) the global CC proteomic profile, squamous cervical cancer proteomic profile (SCC) and cervical adenocarcinoma proteomic profile (CAD).

labeled mixtures were desalted using Sep-Pak C18 Vac cartridges (Waters), pooled, and dried under vacuum. The workflow of the proteomic study is shown in Figure 1.

Peptide pre-fractionation. To increase the confidence level and coverage of the study, we performed a peptide pre-fractionation step using reverse phase (RP) chromatography before the LC-MS/MS. Peptides mixtures were subjected to RP chromatography at high pH on an RP-column (4.9×150 mm) (Waters, USA) using a Binary HPLC Pump (Waters 1525) coupled to a photodiode array detector (Waters 996) set at 210 nm. The buffer system consisted of buffers A (50 mM ammonium formate in water, pH 10) and B (50 mM ammonium formate in 60% acetonitrile, pH 10). The separation was achieved by a gradient elution as follows: 0 to 20 min, 0% B; 20 to 25 min, 0 to 5% B; 25 to 90 min, 5 to 40% B; 90 to 105 min, 40 to 100% B; 105 to 115 min, 100% B; 115 to 120 min, 0% B for re-equilibration. The flow rate was 0.50 ml/min. Thirty fractions were collected; each fraction was dried on Speed-Vac vacuum, and stored at -80°C until LC-MS/MS analysis.

LC-MS/MS. Peptides were resuspended under initial chromatographic conditions and separated using an Agilent 1100 nano pump G2226A coupled to a hybrid triple quadrupole linear ion trap (QTRAP 3200, AB Sciex) equipped with a Nanospray II source and using data dependent acquisition (DDA). The tryptic peptides (8 µl) were desalted and concentrated on a Zorbax column (Agilent 5065-

9913) before analysis on an RP column (Agilent Zorbax 300SB C18, 3.5 µm, 150×0.075 mm). Separation was done at 300 nl/min using a linear gradient. Mobile phase A was water with 0.1% formic acid (v/v). Mobile phase B was acetonitrile with 0.1% formic acid (v/v). The gradient conditions were 100% A (0 min) to 95% (10 min); 100% A (10 min) to 75% (60 min); 70% A (60 min) to 20% (70 min); 20% A (70 min) to 20% (75 min); 20% A (75 min) to 100% (76 min); and 100% A (76 min) to 100% (80 min).

Precursor ion determination was carried out using an enhanced MS scan over a mass range of 400-1,400 m/z at 4,000 amu/s (with no trapping in Q0 and Dynamic fill time) with an ion spray voltage of 2,600 V applied to a Picotip FS360-75-15-N with ion spray gas (nitrogen). The precursor ions collided at Q2 using rolling collision energy (maximum allowed CE=80). Enhanced production scans (MS/MS) were performed in a mass range of 100-1,700 m/z at 4,000 amu/s, and collision voltages were dynamically determined. All precursor ion mass/charge ratios were confirmed with an Enhance Resolution scan.

Protein identification and quantification were carried out using MASCOT search engine (v2.2.07) against Swissport database, setting trypsin as the digestion enzyme and carbamidomethyl-cysteine as fixed modification. N-terminal protein acetylation and oxidation were included as variable modifications. Proteins were identified with a 1% false discovery rate (FDR) <1% (target-decoy). The database used for protein identification was the human reference proteome UP000005640 from UniProt repository,

downloaded on April 30, 2016. Proteins with at least 2 razor-unique peptides identified by MS/MS were considered for quantification. These criteria resulted in 622 proteins identified in at least one experimental replicate.

Normalized spectra were extracted from each CC pool (SqMD16, SqMD16, AdM16, SqMD18, SqMD31, and control) to be analyzed, and the mass spectra were aligned by using MASCOT search engine (v2.2.07). Then the intensity mass peaks in the mass range were selected and exported in CSV file format. Protein analysis was performed for proteins with at least 2 intensity values in each analysis set. Protein abundance was normalized among conditions and the Random Forest method (miss Forest v.4; R package v2.35) was used to impute missing values (15). PCA was implemented in the protein intensity correlation matrix (Facto MineR v2.3; R package v3.5) (16) which led to protein abundance pattern for every CC analysis set (17). To establish whether any component could discriminate among CC analysis sets, the sample scores for each component were plotted. Once the component was found, the significant proteins that possessed discriminating capacity were identified using the squared cosine of the correlation matrix between the components and the proteins (18).

To evaluate the PCA performance on LC-MS/MS data, a t-test (significance level, 0.05) and a fold-change analysis were performed to compare protein abundance ratios among CC analysis sets. The first principal component accounts for as much of the variability in the data as possible, and similarly, each succeeding component explains as much of the remaining variability as possible (19). The first two components were considered for data classification.

SuperExactTest to clarify comprehensive relationships among CC analysis sets. The SuperExactTest (SET) was implemented to visualize comprehensive relationships among CC analysis sets. SET provides the theoretical framework to compute the statistical distributions of multi-set intersections based upon combinatorial theory and a procedure to accurately calculate the exact probabilities of multi-set intersections (20).

Gene set enrichment analysis to elucidate pathways in CC. The mean fold change data of the two experimental replicates were used to produce a preordered ranked gene list data set, then tested for enrichment by Gene set enrichment analysis (GSEA v. 2.0, Broad Institute, Cambridge, MA, USA) (21). Gene sets were generated and modified with biochemical pathway data from the Kyoto Encyclopedia of Genes and Genomes (KEGG; www.genome.jp/kegg). A number of gene sets were differentially enriched ($p < 0.1$ and $FDR < 0.25$) in each cancer tissue compared to control.

Immunohistochemical (IHC) analysis. A representative section was taken from tumor specimens and placed in a 100% formaldehyde solution. The fixed tissue was dehydrated with graded alcohols and embedded in paraffin wax. CC specimen sections (6- μ m thick) were cut and mounted on Super frost plus slides (VWR; Thermo Fisher Scientific). The sections were then deparaffinized with xylene and washed in 100% alcohol. The slides were incubated at room temperature (RT) in 3% (v/v) H_2O_2 in methanol for 30 min to block endogenous peroxidase activity. The slides were then rehydrated through graded alcohols, washed in PBS, pH 7.5 and stained using a Ventana Medical Systems kit (Roche Tissue Diagnostics, Oro Valley, AZ, USA) according to the manufacturer's guidelines from the antibody incubation step.

The antibodies used were: anti-RAB14 1:200 (ab28639; Abcam, Cambridge, UK), anti-RCN3 1:200 (NBP2-13213; Novus Biologicals,

Centennial, CO, USA), and Anti-p16 (725-4713; CINtec® Histology, Roche Diagnostics, Rotkreuz, Switzerland), which was used as a positive control for CC tissues. Staining was assessed in the tumor area using the plug-in IHC profiler from ImageJ.

Plasmid and lentivirus production. Gene expression of RAB14 and RCN3 was carried out using plasmid expression vectors. Viral particles containing the RAB14 sequence gene were produced by a co-transfection using the plasmids psPax2 (12260; Addgene), pMD2G (Addgene no. 12259), pL-SIN-EF1a-EGFP (21320; Addgene), and RAB14 WT (plasmid #61493; gift by Dr. Curt Civin, Addgene). Viral particle production was performed in HEK 293T cell line by using lipofectamine 3000 (L30000015; Invitrogen). According to the product instructions, viral particles produced in cultured cells were harvested at 48 h post-transfection and cleared by a low-affinity protein filter (0.45 μ m pore size) and frozen at -70°C until further use. pCDNA 3.1 red fluorescent protein (RFP) (13032; Addgene) RCN3 was generated using shrimp alkaline phosphatase (ALP) (783901000UN; Thermo Fisher) to avoid plasmid recirculation, followed by T4 DNA ligase cloning (EL0011; Invitrogen). pCDNA3.1RFP-RCN3 plasmid was transfected into *E. coli* DH5 α and selected by carbenicillin resistance, grown in Luria-Bertani (LB) broth media and verified by detection of RCN3 by PCR. pCDNA 3.1 plasmid purification was performed using the Genomic DNA Purification Kit (K0512; Thermo Fisher) and the purified pCDNA3.1 RFP-RCN3 was stored at -70°C until use in transfection.

Cell transfection. HeLa cells were grown until reaching 80% cell confluence using RPMI 1640 5% fetal bovine serum. Transfection with the plasmids mentioned above was performed using lipofectamine 3000 (L30000015; Invitrogen) in p60 cell culture plates. In addition, cells were transfected with pCDNA empty vector (Mock), as control. After 48 h post-infection/transfection, cells were harvested and washed in PBS pH 7.5, counted and inoculated in immunodeficient mice.

Tumor xenograft mouse model. In this study, 4-6 old week old nude female athymic BALB/c nu/nu mice were used. The Bioethics Committees of UNAM and INCAN approved the protocol used for animal handling in this study. To develop tumor xenografts in mice, 2×10^6 HeLa cells transfected with RAB14 or RCN3 and injected subcutaneously in one flank of nude mice. The signs of tumor formation were observed daily and measured every week in triplicate. Control animals were injected with wild-type HeLa cells in serum-free cell culture media. Tumor volume was measured using the Attia-Weiss formula: $a \times b^2 \times 0.4$, where a = largest diameter and b = smallest diameter of tumor. After 8 weeks, mice were sacrificed, and the subcutaneous tumors were removed, photographed, fixed in 4% formaldehyde and embedded in paraffin. A representative section was taken from tumors induced by RAB14 or RCN3 HeLa transfected cells to measure the level of protein expression using wild-type HeLa cells tumors as a control.

Results

Patients and histopathological diagnosis. To perform quantitative proteomics, the histopathological diagnosis of each sample was performed by microscopic examination of a 5- μ m thick section of each research tissue sample. The eighteen samples were classified into 6 groups (SqPD16,

Table I. Statistically significant protein changes were classified in upregulated, downregulated or non-differentially expressed proteins in CC, SCC and CAD analysis pools.

Protein expression	Cancer type		
	CC	SCC	CAD
Up-regulated	40	60	41
Non-differentially expressed	571	552	565
Down-regulated	11	10	16

CC: Cervical cancer; SCC: squamous cervical cancer; CAD: cervical adenocarcinoma.

SqMD16, AdMD16, SqMD18, SqMD31 and control), each of which consisted of 3 samples, as described in Materials and Methods.

Proteomics. Each group consisted of 3 individual samples, and all 18 samples were analyzed. A total of 622 proteins identified and quantified in both replicates (Supplemental Table I) were processed by PCA, a statistical method designed to alleviate the degree of complexity in high-dimensional data but allowing the retention of trends and patterns (19), which is done by transforming the data into fewer dimensions that act as feature summaries.

According to PCA results, the component scores of spectra were plotted for Dimension 2 (Dim2; Y-axis) and Dimension 1 (Dim1; X-axis). In PCA, the projections indicated the separation of the control group (non-cancerous, HPV-negative cervical tissue) from the other groups (HPV+, cervical cancer tissue). SqMD16 and SqPD16 tended to be close and SqMD31 and SqMD18 suggested certain tumor proteins in common. In addition, the highest correlation among all CC analyzed groups was observed between SqMD16 and SqPD16 samples, suggesting HPV16 contribution was similar in moderately differentiated SCC samples and SCC poorly differentiated. Finally, we discovered a pronounced difference between SCC (SqPD16, SqMD16, SqMD31 and SqMD18) and CAD proteomic profile, which highlights the differences at the proteome level between SCC and CAD (Figure 2). The proteins obtained from the PCA analysis were also classified according to gene ontology (GO)-biological process (BP) classification. The BP analysis showed that 25% of the proteins identified and quantified belong to the exocytosis pathway (regulated exocytosis and exocytosis). Protein binding was the most predominant category for proteins in the molecular function classification, followed by structural molecule activity. For cell component classification proteins identified in CC, more than a half of the classified proteins were related to vesicle and extracellular space components (Figure 3). Next, PCA was used to estimate the similarity between them. Statically significant proteins were retrieved

Figure 2. The biplot gives an indication about clustering and trends present in the protein profiles. Scores of similar cervical cancer (CC) pools will tend to form clusters whereas dissimilar samples will be located at greater mutual distances. The results demonstrate that SCC protein profiles constitute a cluster; SqMD31 and SqMD18 are closer relatives than SqMD16 and SqPD16 although they both belong to SCC profile. AdMD16 is located at a larger distance from the rest of CC groups. Control is located near 10 over the dimension 2 (Dim2) axis, far from the CC groups.

over (1.9) or below (−1.9) fold-change compared to control. Also SET was completed to visualize up-regulated and down-regulated proteins in CC multi-set intersections. Results are shown in Figure 4 and Table I. CAD showed a more specific proteomic pattern compared to SCC and CC. Out of 622 proteins, 597 were included in GSEA, considering only those collapsed in the analysis software data set.

GSEA to elucidate pathways in CC. The fold-change data, obtained from 2 experimental replicates were used to formulate a gene list data set, further tested by GSEA (GSEA v. 2.0, Broad Institute, Cambridge, MA, USA) (19). Gene sets were generated and modified using biochemical pathway data from the Kyoto Encyclopedia of Genes and Genomes (KEGG; www.genome.jp/kegg). Several sets of genes were differentially enriched ($p < 0.1$ and $FDR < 0.25$) in each CC tissue compared to the control. The results obtained from GSEA provided 39 sets of genes ($p\text{-value} \leq 0.1$ or $FDR \leq 0.25$) (Supplemental Table II and Supplemental Figure 1), of which 21 gene sets ($p\text{-value} \leq 0.1$ and $FDR \leq 0.25$) were chosen according to the GSEA parameters and criteria. These gene sets are statistically enriched in at least one CC cancer group. Next, the down-regulated and up-regulated pathways from GSEA were classified (Table II) including main proteins for each set. Focal adhesion, protein digestion and absorption, papillomavirus, and extracellular matrix receptor (ECM)

Figure 3. A total of 622 proteins identified and quantified by isobaric tags for relative and absolute quantitation (ITRAQ) were grouped according to gene ontology classification by biological process. Regulated exocytosis and exocytosis together accounted for 25% of total classification. CC, Cervical cancer.

interaction were the most the overrepresented pathways in GSEA. Compared to the control, one of the most up-regulated pathways in CC was calcium signaling; where voltage dependent anion channel 1 (VDAC-1) was identified as a critical interactor (Supplemental Table III). On the other hand, the ECM-receptor interaction was one of the most down-regulated pathways found in CC compared to control. This result is supported by the downregulation of collagens (COL6A3, COL6A1, COL14A1, and COL6A2) that we experimentally discovered in our study. Statistically significant proteins obtained by PCA and a fold-change analysis were classified into 3 groups: i) proteins differentially expressed in SCC compared to control (Table III), ii) proteins differentially expressed in CAD compared to control (Table IV), and iii) common proteins between SCC and CAD compared to control (Table V). The information

included in the tables was obtained from scientific reports that considered protein participation in the hallmarks of cancer, and BP was annotated to the proteins that no reports of participation in cancer were found. SCC_CTRL (Table III) showed that almost 50% of the proteins included are related to activating invasion and metastasis. The comparison of SCC with control showed that 22 proteins were overexpressed: activating invasion and metastasis was the leading category with 9 proteins (CALL5, ZN185, GT251, S10AE, K1C15, FLNB, K2C6C, NEBU, YBOX3).

Compared to the control (Table IV), the classification of CAD proteins showed resisting cell death as the main category of proteins obtained. Protein S100-A9 (S10A9), ribosome-binding protein 1 (RRBP1), protein S100-A13 (S10AD), histone H2AX (H2AX) and protein S100-A12 (S10AC) were classified in the main category. Endoplasmic reticulum

Figure 4. SuperExactTest (SET) was used to establish multi-set intersections among CC analysis sets, for up-regulated (UR) and down-regulated (DR) proteins. A total of 17 UR and 10 DR proteins were common in all the three analysis sets (CC, SCC and CAD). The color intensity of the bars represents the p -value significance of the intersections. CC, Cervical cancer; SCC, squamous cell cancer; CAD, cervical adenocarcinoma.

ribosomal binding protein 1 (RRBP1) is a transporter located in the rough endoplasmic reticulum (ER). Twenty-seven proteins were shared between SCC and CAD compared to control. Resisting cell death and activating invasion and metastasis were the main common cell function categories found in SCC and CAD. The down-regulated proteins in SCC and CAD compared to control were related to cell adhesion. Furthermore, the 10 kDa heat shock protein (CH10) was up-regulated in SCC and CAD compared to control.

In this study, prosaposin (SAP) was overexpressed in general terms in CC samples compared to control. However, we emphasize the greater overexpression of SAP at the proteomic level in CAD compared to control. Considering this difference, we hypothesized that SAP might be related to promoting the survival of tumor CC cells under stress conditions, contributing to the worse clinical outcome in CAD compared to SCC.

Reticulocalbin 3 (RCN3) was overexpressed in both CAD and SCC, compared to control tissue. For this reason, we decided to perform further *in vivo* analysis to investigate the role in CC development since the evidence associated with this protein in CC it is still scarce.

IHC analysis. IHC analysis was performed to verify protein expression in 9 CC tumor samples. These samples were cut and stained with hematoxylin and eosin, p16, RAB14, and RCN3 antibodies. IHC slides were evaluated using IHC

profiler plug-in, and the result was positive for 6/9 samples analyzed for p16 IHC (considered as a positive control for CC), 6/9 positive for RAB14 protein, and 5/9 positive tissue samples for RCN3 protein. Three different samples are shown in Figure 5.

RAB14 and RCN3, two ER proteins up-regulated in CC, were selected to further study their role in tumor biology. RAB14 was overexpressed in SqPD16 in 2 replicates, and RCN3 was overexpressed in CC analysis groups compared to control tissue by PCA data. Evidence provided at the proteomics level led us to perform *in vivo* studies considering RCN3 was overexpressed (1.94-fold-change) in CC compared to the control (Table V), and RAB14 was overexpressed (3.12-fold-change) in SqPD16 compared to the control (Supplemental Table I).

Tumor xenograft model. RAB14 and RCN3 HeLa transfected cells were separately inoculated subcutaneously into female nu/nu mice. Tumor formation was visible on day 16; HeLa RCN3 tumors showed a moderate effect on tumor growth development until day 47. In contrast, HeLa RAB14-expressing cells showed effects on tumor size from the beginning to the end of the tumor formation experiment (Figure 6A and B). The expression of RCN3 and RAB14 proteins in the xenograft tumors was evaluated by Western blot by selecting the smallest tumor and the largest tumor of each (Figure 6D). For RAB14 protein, Western blot analysis showed that tumor volume was positively correlated with RAB14 protein expression (Figure 6E). RCN3 tumors showed higher RCN3 protein expression over vector and WT HeLa control cells; however, a moderate relation was found between RCN3 protein expression and tumor size increment was visible until day 47. RAB14-inoculated mice developed larger tumors than those with control cells. The increase in tumor size in RAB14 was most pronounced around day 35 and was maintained until day 47, showing and confirming the role of RAB14 in tumorigenesis, as shown in Figure 6.

Discussion

CC remains one of the most common types of cancer in women worldwide. Although CC early detection and public health programs have decreased the number of cases and mortality rates, the response to treatment in CC variants remains poor. Detection systems such as the Pap and HPV DNA test have reduced the incidence and mortality of CC. However, CAD has shown a poor prognosis for patients and a greater probability of distant recurrence than SCC (76). In this study, global CC, SCC, and CAD proteomics profiles were obtained by comparing CC tissues with control tissue. Six hundred twenty-two proteins identified and quantified by LC/MS were processed by PCA, which allowed us to group and obtain global proteomic patterns that we classified as CC,

Table II. Statistically significant enrichment pathways in cervical cancer according to gene set enrichment analysis (GSEA).

Cervical cancer tissue type	Pathway	p-Value	Involved proteins
CC (SCC & CAD)	Cell cycle	<0.0001	ARHGDIB CALML3
	Neurotrophin signaling pathway	0.0011	CAMP SFN LYZ
	Oocyte meiosis	0.0012	VDAC1 VDAC3 YWHAB
	Salivary secretion	0.0305	YWHAE YWHAH
	Calcium signaling pathway	0.0277	YWHAZ YWHAQ
			CALML5
	Protein digestion and absorption	<0.0001	COL1A1 COL1A2
	ECM-receptor interaction	<0.0001	COL2A1 COL6A1
	Papillomavirus	<0.0001	COL6A2 COL6A3
	Gap junction	<0.0001	PPP1CC TUBB6
	Focal adhesion	<0.0001	TUBA4A COL14A1
			VCL FLNA TUBB
			TUBA1A TUBB2B
			TUBA1B TUBB4B
			TUBA1C
SCC	Cell cycle	<0.0001	ARHGDIB CALML3
	Oocyte meiosis	<0.0001	CAMP CTSD SFN
	Neurotrophin signaling pathway	<0.0001	HSP90AA1 HSP90AB1
	Tuberculosis	0.0160	HSPD1 LSP1 STAT1
	Plant-pathogen interaction	0.0224	HSP90B1 YWHAB
			YWHAE YWHAH
			YWHAZ YWHAQ
			CALML5 CYCS
	Protein digestion and absorption	<0.0001	COL1A1 COL1A2
	Papillomavirus	<0.0001	COL2A1 COL6A1
	ECM-receptor interaction	<0.0001	COL6A2 COL6A3
	Gap junction	<0.0001	TUBA4A COL14A1
	Focal adhesion	<0.0001	TUBA1A TUBA1B
			TUBB4B TUBB6
			TUBA1C TUBB
SCC compared to CAD	Oocyte meiosis	<0.0001	ALDOA ARHGDIB SFN
	Cell cycle	0.002	GPI PFKL PFKP PGM1
	Pentose phosphate pathway	0.002	TUBA4A YWHAB
	Glycolysis/gluconeogenesis	0.003	YWHAE YWHAH
	Gap junction	0.008	YWHAZ TUBA1A
			TUBA1B TUBB4B
			YWHAQ CALML5 TUBB6
			TUBA1C TUBB TUBB2B
	Systemic lupus erythematosus	0.013	APOA1 COL1A1 COL1A2
	Protein digestion and absorption	0.012	COL2A1 ELANE H2AFX
	African trypanosomiasis	0.046	HBA2 HBB COL14A1
			HIST1H3F HIST1H2AG
			H2AFY H2AFV
			ATP5B ATP5D
			ATP6V1E1 ATP5O
CAD	Systemic lupus erythematosus	0.001	CALML3 CAMP
	Cardiac muscle contraction	0.004	ELANE H2AFX
	Oxidative phosphorylation	0.049	HSPD1 LSP1 NDUFS4
	Tuberculosis	0.076	SCP2 SOD1 TPM1
	Peroxisome	0.084	TPM2 TPM3 TPM4
			HIST1H3F HIST1H2AG
			H2AFY NDUFA13
			CYCS H2AFV
	Protein digestion and absorption	<0.0001	A2M SERPINC1 CPAMD1
	Papillomavirus	<0.0001	COL2A1 COL6A1
	Gap junction	<0.0001	COL6A2 COL6A3
	ECM-receptor interaction	0.003	KNG1 PKM PLG
	Complement and coagulation cascades	0.011	TUBA4A COL14A1
			TUBA1A TUBA1B
			TUBB4B TUBB6 TUBA1C
			TUBB TUBB2B

CC: Cervical cancer; SCC: squamous cervical cancer; CAD: cervical adenocarcinoma.

Table III. *Differentially expressed proteins in SCC.*

Number	Name	CC_CTRL ¹	p-Value ²	SCC_CTRL ¹	p-Value ²	CAD_CTRL ¹	p-Value ²	SCC_CAD ¹	p-Value ²	Cell function
1	CALL5	1.829	0.067	2.073	0.041	0.431	0.747	1.642	0.049	Activating invasion and metastasis (21-23)
2	OSTF1	1.888	0.001	2.062	0.000	1.385	0.046	0.677	0.025	Ossification
3	COTL1	1.801	0.049	1.992	0.039	0.874	0.504	1.118	0.191	Sustaining proliferative signaling (22-24)
4	ZN207	1.895	0.000	1.991	0.000	1.758	0.026	0.233	0.288	Cell division
5	1433B	1.794	0.000	1.988	0.000	0.716	0.387	1.273	0.041	Resisting cell death (23-25)
6	ZN185	1.678	0.082	1.981	0.072	1.212	0.293	0.769	0.357	Activating invasion and metastasis (24-26)
7	1433G	1.750	0.000	1.936	0.000	0.684	0.415	1.252	0.056	Resisting cell death (23-25)
8	PERE	1.602	0.048	1.933	0.037	0.837	0.593	1.097	0.342	Tumor promoting inflammation (25-27)
9	GT251	1.837	0.118	2.063	0.119	0.673	0.660	1.390	0.191	Activating invasion and metastasis (26-28)
10	S10AE	1.898	0.003	2.040	0.003	1.571	0.057	0.469	0.162	Activating invasion and metastasis (27-29)
11	K1C15	1.708	0.010	1.997	0.006	0.630	0.637	1.367	0.165	Activating invasion and metastasis (28-30)
12	FLNB	1.737	0.000	1.960	0.000	1.032	0.308	0.929	0.158	Activating invasion and metastasis (29-31)
13	GLU2B	1.807	0.000	1.921	0.000	1.663	0.024	0.258	0.306	Cellular protein metabolic process
14	1433F	1.738	0.000	1.921	0.000	0.711	0.404	1.210	0.063	Resisting cell death (23-25)
15	PLIN3	1.767	0.000	1.970	0.001	1.228	0.032	0.742	0.021	Resisting cell death (30-32)
16	K2C6C	1.654	0.001	1.935	0.001	0.683	0.544	1.253	0.143	Activating invasion and metastasis (28-30)
17	NONO	1.896	0.001	1.932	0.003	1.585	0.066	0.347	0.236	Sustaining proliferative signaling (31-34)
18	PSA2	1.673	0.055	1.904	0.051	0.776	0.546	1.128	0.230	Resisting cell death (33-35)
19	NEBU	1.824	0.008	2.065	0.006	1.303	0.310	0.762	0.323	Activating invasion and metastasis (34-36)
20	TBCA	1.808	0.000	1.951	0.000	1.456	0.023	0.495	0.022	Resisting cell death (35-37)
21	YBOX3	1.756	0.000	1.948	0.000	1.136	0.083	0.811	0.020	Activating invasion and metastasis (36-38)
22	1433T	1.741	0.000	1.930	0.000	0.672	0.394	1.258	0.039	Resisting cell death (23-25)

¹Protein expression fold-change; ²p-Value according to Gene Set Enrichment Analysis. CC: Cervical cancer; SCC: squamous cervical cancer; CAD: cervical adenocarcinoma; CTRL: control.

SCC, and CAD. This analysis aimed at finding common protein patterns in CC and specific ones in SCC and CAD. BP analysis classified 25% of proteins as proteins related to exocytosis and 24% as components of extracellular vesicle and organelle components. The experimental data that we obtained emphasizes the importance of endo/exocytosis proteins in CC. Recent evidence has suggested that altered protein trafficking routes/networks are preferentially involved in acquiring pro-metastatic traits (77). Endosomal recycling has been reported to play a central role in integrating circuits linked to metastasis and resistance to cell death, the two most overrepresented protein categories in CC found in our study. In particular, the family of Ras-related proteins is involved in acquiring metastatic traits and resistance to apoptosis mediated by endosomal recycling (78).

From the set of 622 proteins identified and quantified in this study (Supplemental Table I), 597 were subjected to GSEA, and were classified according to experimental evidence reported in the bibliography. Differentially expressed SCC proteins showed activating invasion and metastasis as the primary cell function in SCC (Table III), resisting cell death for CAD differentially expressed proteins (Table IV), and resisting cell death and activating invasion and metastasis as the two common cell function categories in SCC and CAD compared to control (Table V).

RRPB1 has been shown to be overexpressed in lymph node metastasis in CC patient samples (39) (Table IV). In addition, RRPB1 is involved in endoplasmic reticulum stress (ERS) release in lung cancer, as a response to increasing tumor cell proliferation functions like protein synthesis,

Table IV. Differentially expressed proteins in CAD.

Number	Name	CC_CTRL ¹	p-Value ²	SCC_CTRL ¹	p-Value ²	CAD_CTRL ¹	p-Value ²	SCC_CAD ¹	p-Value ²	Cell function
1	S10A9	1.726	0.001	1.546	0.001	2.441	0.163	-0.895	0.243	Resisting cell death (37-39)
2	RRBP1	1.597	0.018	1.558	0.039	2.040	0.206	-0.483	0.444	Resisting cell death (38-41)
3	S10AD	1.575	0.054	1.578	0.089	2.153	0.035	-0.575	0.040	Resisting cell death (40-42)
4	H2AX	1.489	0.046	1.397	0.092	2.149	0.065	-0.752	0.000	Resisting cell death (41-44)
5	CEAM5	1.650	0.060	1.338	0.147	2.600	0.313	-1.262	0.410	Activating invasion and metastasis (43-45)
6	GPVI	1.807	0.014	1.732	0.032	2.322	0.287	-0.589	0.520	Activating invasion and metastasis (44-47)
7	S10A6	1.470	0.000	1.327	0.001	2.026	0.205	-0.700	0.358	Activating invasion and metastasis (46-48)
8	ELNE	1.853	0.001	1.870	0.001	2.125	0.196	-0.255	0.558	Tumor promoting inflammation (47-50)
9	CEAM8	1.310	0.141	1.085	0.246	2.014	0.304	-0.929	0.451	Tumor promoting inflammation (49-51)
10	S10AC	1.107	0.091	0.927	0.211	1.936	0.324	-1.009	0.415	Resisting cell death (40-42)
11	CYC	1.610	0.007	1.577	0.011	1.928	0.096	-0.351	0.337	Deregulating cellular energetics (45-47), (50-52)
12	K2C8	1.410	0.023	1.507	0.075	1.910	0.021	-0.403	0.066	Activating invasion and metastasis (51-53)
13	CAMP	1.693	0.052	1.606	0.096	2.339	0.161	-0.732	0.308	Tumor promoting inflammation (52-55)
14	CYTA	1.569	0.004	1.346	0.026	2.336	0.120	-0.990	0.198	Evading growth suppressors (54-56)

¹Protein expression fold-change; ²p-Value according to Gene Set Enrichment Analysis. CC: Cervical cancer; SCC: squamous cervical cancer; CAD: cervical adenocarcinoma; CTRL: control.

folding, and secretion. This was shown by the expression of the ectopic protein RRPB1 in non-small cell lung cancer cell lines when ERS was induced using tunicamycin (40). Furthermore, it has been reported that RRPB1 is highly expressed in prostate cancer and correlates with poor prognosis, and thus has been suggested as a potential biomarker in PCa (79) and ovarian cancer (80). Patients with higher expression of RRPB1 have been reported to have worse overall survival; hence RRPB1 has also been suggested as a target for epithelial ovarian cancer treatment (80).

RCN3 is a Ca²⁺ binding protein in the secretory pathway and is located in the ER (81). To date, the evidence of RCN3 in relation to cancer development is insufficient compared to reticulocalbin 1 (RCN1). RCN1 is a member of the Ca²⁺ binding protein family expressed in the highly metastatic prostate cancer cell line PC-3 (82). RCN1 mRNA expression increased in the highly invasive breast cancer cell line MDA-MB-435, compared to poorly invasive MCF-7 (83). Furthermore, RCN1 has been suggested as a potential biomarker for renal cell carcinoma when discovered by proteomic approach and validated by IHC in 21 of 24 renal cell cancer paired tissue samples (84). In prostate cancer, RCN1 expression arose in bone marrow endothelial cells (BMECs), increased in the presence of tumor necrosis factor α (TNF- α), a pro-inflammatory cytokine often expressed in prostate cancer

patients with very advanced metastasis (85). RCN2 is another member of the reticulocalbin family which, when detected overexpressed in hepatocellular cell cancer (HCC), has been involved in cell proliferation and tumor growth, presumably by regulating epidermal growth factor receptor (EGFR) activation and the extracellular signal-regulated kinase (ERK) pathway (86). RCN1 and RCN2, in contrast with RCN3, have been extensively studied. This notion guided us to explore the role of RCN3 in oncogenesis, especially in CC.

In the present study, we found that the expression of the RCN3 protein was upregulated in all CC analysis sets when compared to the control (Table V). IHC analysis showed positive expression of RCN3 in 5/9 samples. Moreover, RCN3-overexpressing HeLa cells inoculated in female nu/nu mice induced a moderate increased tumor growth compared to HeLa mock cells, at day 47. RCN3 has been identified as a negative regulator of collagen production in cardiac fibrosis. CRISPR/Cas9-mediated activation of RCN-3 decreased collagen production in human cardiac fibroblasts (62). In this study, down-regulation of collagen type I alpha 1 chain (COL1A1) and collagen alpha-2(I) chain (COL1A2) was observed in CC samples, compared to control. Previous studies have shown that collagen expression depends on the tumor stage. Specifically, normal breast fibroblasts in co-cultures with breast cancer cell lines derived from late-stage breast cancer

Figure 5. Immunohistochemical (IHC) assay for detection of RAB14 and RCN3 protein expression; p16 was considered positive control), RAB14 and RCN3. Six out of 9 samples were positive for RAB14 protein and 5 out of 9 slides from CC samples were positive to RCN3 protein. H&E, Hematoxylin-eosin.

(MDA-MB-231, MCF-7, T47D, and ZR-75-1) have been shown to exhibit a greater than 50% reduction in collagen protein. Furthermore, in breast cancer tissue samples, Northern analysis has revealed that collagen mRNA levels were elevated in stage I tumors compared to adjacent normal breast tissues, while decreased in stage II and III breast tumors (87). Therefore, we suggest that RCN3 may participate as a negative regulator of collagen production in CC and modulates the dynamic collagen concentration from the early to late stages of tumor development. In the early stages, the collagen concentration tends to increase compared to the adjacent extracellular matrix (ECM), this protein in cancer has been

studied, and evidence shows that collagen type I promotes resistance to apoptosis in fetal calf serum-starved MCF-7 and T-47D cells (88). Furthermore, E-cadherin gene expression has been found to be downregulated by collagen type I and type III in pancreatic cancer cell lines (89). In addition, pathological conditions such as cancer cell proliferation and metastatic events have been associated with defective E-cadherin-mediated cell-cell adhesion (90). Therefore, RCN3 may be implicated in the modulation of collagen production and E-cadherin gene expression in CC cells.

A quarter of all proteins identified and quantified in CC are related to exocytosis. Recent lines of evidence support

Table V. Common proteins between SCC and CAD.

Number	Name	CC_CTRL ¹	p-Value ²	SCC_CTRL1	p-Value ²	CAD_CTRL1	p-Value ²	SCC_CAD1	p-Value ²	Cell function
1	ICAL	2.575	0.000	2.638	0.000	2.685	0.174	-0.047	0.774	Activating invasion and metastasis (55-57)
2	EFHD2	2.190	0.000	2.342	0.000	1.916	0.078	0.426	0.166	Resisting cell death (56-58)
3	SAP	2.103	0.001	2.029	0.007	2.810	0.019	-0.781	0.002	Resisting cell death (57-59)
4	IF4H	2.029	0.014	2.047	0.009	2.142	0.032	-0.095	0.299	Resisting cell death (58-61)
5	ML12B	1.971	0.000	2.103	0.001	1.993	0.001	0.110	0.898	Muscle contraction
6	RS28	2.171	0.000	2.285	0.000	1.953	0.072	0.333	0.325	Cytoplasmic translation
7	PRTN3	2.137	0.013	2.223	0.008	2.088	0.089	0.135	0.851	Antimicrobial humoral response
8	CH10	2.037	0.000	1.910	0.000	2.663	0.012	-0.753	0.000	Activating invasion and metastasis (60-62)
9	RCN3	1.943	0.042	1.940	0.046	1.975	0.192	-0.034	0.938	Activating invasion and metastasis (61-64)
10	GVIN1	1.919	0.000	1.974	0.000	2.039	0.092	-0.065	0.561	Tumor promoting inflammation (63-65)
11	PERM	2.340	0.005	2.036	0.020	3.069	0.020	-1.034	0.018	Tumor promoting inflammation (64-67)
12	TPD54	2.320	0.000	2.323	0.000	2.521	0.090	-0.199	0.397	Deregulating cellular energetics (66-69)
13	SRSF2	2.299	0.090	2.396	0.074	1.991	0.131	0.406	0.154	mRNA processing
14	HEBP2	2.301	0.001	2.361	0.002	2.535	0.012	-0.173	0.197	negative regulation of mitochondrial membrane potential
15	TPM3L	2.155	0.000	2.295	0.000	1.983	0.048	0.312	0.262	Actin filament organization
16	ANXA3	2.236	0.011	2.430	0.007	1.951	0.059	0.479	0.201	Inducing angiogenesis (68-71)
17	MARCS	2.795	0.000	3.010	0.000	2.296	0.260	0.714	0.347	Resisting cell death (70-73)
18	MIME	-2.668	0.288	-2.778	0.283	-2.074	0.284	-0.704	0.695	Negative regulation of smooth muscle cell proliferation
19	COEA1	-2.670	0.303	-2.704	0.302	-2.624	0.291	-0.079	0.965	Cell-cell adhesion
20	DESM	-2.558	0.291	-2.456	0.291	-2.595	0.272	0.139	0.973	Inducing angiogenesis (72-74)
21	KCRB	-2.035	0.000	-1.972	0.000	-2.108	0.071	0.136	0.893	Deregulating cellular energetics (73-75)
22	CO6A3	-1.950	0.230	-1.959	0.233	-2.349	0.183	0.390	0.627	Cell adhesion
23	NQO1	-2.056	0.076	-1.972	0.074	-2.693	0.075	0.722	0.040	Cell redox homeostasis
24	CO6A1	-2.209	0.333	-2.140	0.340	-2.908	0.294	0.768	0.518	Cell adhesion
25	CO6A2	-2.387	0.167	-2.364	0.168	-3.312	0.145	0.948	0.219	Cell adhesion
26	TGM2	-2.501	0.090	-2.201	0.087	-3.232	0.094	1.032	0.038	Activating invasion and metastasis (74-76)
27	ALBU	-2.182	0.085	-2.098	0.086	-3.099	0.056	1.001	0.202	Negative regulation of apoptotic process

¹Protein expression fold-change; ²p-Value according to Gene Set Enrichment Analysis. CC: Cervical cancer; SCC: squamous cervical cancer; CAD: cervical adenocarcinoma; CTRL: control.

the participation of exocytosis in the acquisition of metastatic traits and resistance to apoptosis through Ras-related protein family as endosomal recycling mediators. RAB14 belongs to a family of regulator proteins that orchestrate vesicle trafficking events, including vesicle formation, vesicle movement, and vesicle binding to their target membrane (91). RAB14 was overexpressed in SqPD16 compared to control, and 6/9 CC samples were positive to RAB14 by

IHC. RAB14 acts as a molecular switch between inactive and active states based on its binding to guanosine diphosphate (GDP) and guanosine triphosphate (GTP), respectively, to activate downstream pathways. RAB14 is up-regulated in gastric cancer tumor tissues compared to adjacent non-tumor tissues (92). At post-translational level (miRNAs), RAB protein has been suggested as an oncogene in non-small cell lung carcinoma (NSCLC) due to the

Figure 6. Tumor xenograft model: RAB14- and RCN3- overexpressing HeLa cells were separately inoculated subcutaneously into female nu/nu mice. Tumor growth curves measured for wild type, RAB14, RCN3, or vector expressing HeLa cells (A). Bar graph showing tumor volume; values are presented as mean $\pm$ SD (B). Photos of the excised tumors from each group (C). The expression of RAB14 and RCN3 was analyzed by western blot assay in smallest tumor (ST) and biggest tumor obtained (BT); b-actin was used as an internal control (D). Relative protein expression of RAB14 (E) and RCN3 (F) was measured by western blot assay considering tumor volume; b-actin was used as internal control.

frequent loss of its negative miRNA regulators miR-451 and miR-338-3p (93, 94). Evidence provided by ectopic expression of miRNA-451 showed negative effects on *in vitro* proliferation and colony formation of NSCL cells and the development of tumors in nude mice by enhancing apoptosis. Ectopic expression of miR-451 inhibited RAB14 protein expression, and RAB14 restoration partially attenuated tumor suppressor function of miR-451 in NSCLC. RAB14 may be a critical regulator for cell proliferation in cancer (93). Furthermore, analyses performed on RAB14-positive vesicles confirmed that 38 cargo proteins were directly involved in metastasis. It was proposed that RAB14

participates as an active metastasis mediator by facilitating endosomal recycling (95).

In our xenograft murine model, RAB14 overexpression showed a positive correlation with tumor growth. According to previous experiments in 3T3-L1 adipocytes, optimal levels of RAB14 direct the traffic of glucose by the transporter type 4 (GLUT4). This was demonstrated when depletion of endogenous RAB14 by shRNA-mediated knockdown inhibited sorting of GLUT4 to an internal compartment(s) by means of vesicles (96). GLUT4 belongs to a family of glucose transporter isoforms distributed in different organs. GLUT1 and GLUT3 are found in neurons, astrocytes, fat, and muscle tissues (97),

GLUT2 in enterocytes, kidney, and liver cells (98). GLUT4 is found in muscle and fat cells and is responsible for 80% of glucose intake in skeletal muscle (99). RAB14 overexpression in HeLa cells suggest RAB14 may orchestrate glucose recruitment by translocation of more GLUT4 glucose transporters from intracellular compartments (Golgi membranes and endosomes) to the cell surface, thus sustaining abnormal cellular energy that contributes to tumor growth (100) as we experimentally observed. This new role for RAB14 in cervical cancer may provide a new direction in prognosis or therapy.

Conclusion

RAB14 and RCN3 were indicated as potential biomarkers and therapeutic targets in CC. SCC and CAD proteomic profiles offer valuable information that may serve to identify novel biomarkers associated with disease prognosis and new potential therapeutic targets for the treatment of CC.

Supplementary Material

Supplemental Figure 1, Supplemental Tables I, II, and III can be consulted in the following link: <http://sinik.ccg.unam.mx/PGFP/Ramirez.AR2021.No399-R/>

Conflicts of Interest

The Authors declare that the research was carried out in the absence of any commercial or financial relationship that could potentially be considered as conflicts of interest.

Authors' Contributions

SEG conceptualized the research. ART, JG, AGC and SEG conceived and designed the experiments. ART, SC, JG, GR, and HAVG performed the experiments. ART, LGC and ESB analyzed the data. SEG and AGC contributed resources and equipment. ART and SEG wrote the paper. All Authors read and approved the final manuscript.

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Availability of Data and Materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

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