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ORIGINAL ARTICLE

Protein Expression Analysis in Uterine Cervical Cancer for Potential Targets in Treatment

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Abstract

Specific markers in lesions of the human uterine cervix cancer (UCC) are still needed for prognostic, diagnostic and/or therapeutic purposes. In this study we evaluated key molecules at protein level between normal epithelium, cervical intraepithelial neoplasia (CIN1–3) and invasive cancer of a group of molecules previously reported at mRNA level. For that purpose, human formalin-fixed paraffin embedded tissue microarrays (TMAs) were constructed containing 205 Mexican tissue core specimens. Immunohistochemistry and quantitative analysis of histological staining was performed against twenty-two distinct proteins for each core and the processing platform ImageJ. In the progression of the disease we found key statistical differences for the proteins SEL1, Notch3 and SOCS3. High expressions of SEL1L, Notch3 and SOCS3 have potential value to increase the prognostic of UCC in combination with markers such as $p16^{INK4a}$. This study identified key drivers in cervical carcinogenesis that should be evaluated for the development of UCC therapies.

Keywords Uterine cervical cancer · SEL1L · Notch3 · SOCS3 · Immunohistochemistry · Tissue microarray

Background

Uterine cervical cancer (UCC) is a major cause of death from gynecologic cancer [1] and the third leading cause of female cancer mortalities worldwide. Despite extensive efforts to diminish UCC through screening programs Latin America has one of the highest incidences and mortality rates in the world. CC progresses slowly from pre-invasive cervical intraepithelial neoplasia (CIN) or adenocarcinoma in situ to squamous cell carcinoma (SCC) or adenocarcinoma, respectively. At present, Pap smear triage is the key for early detection of premalignant cervical lesions and cancer in asymptomatic women; however, concerns about poor reproducibility and sensibility have led to the search for specific molecules expressed at the very early stage of the disease.

Numerous studies have reported promising biomarkers; nevertheless, few have been completely evaluated and validated. An increasing number of data have been released to understand the mechanisms that trigger the development and progression to SCC through several platforms and at each stage of the disease. Some of the most recent data from patient samples includes the screening of serum proteins, long non-coding RNAs, microRNAs and gene expression profiles.

The abovementioned studies, although carefully conducted, particularly those reporting mRNA or cDNA profiles, have critical constraints such as the lack of information about the eventual expression and/or posttranscriptional modifications processed for the decisive effectors in the disease. This reality has led global biomarker research to protein discovery or protein profile expression studies. This, combined with the complexity that causes the molecular mechanisms of UCC suggests that multiple markers are required for clinically useful molecular diagnostics assays [2] to improve the accuracy of the detection [3].

Recently, we identified and validated a number of genes at mRNA level related to the Notch signaling pathway and

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tumorigenesis in each chronological stage of the disease [4]. The Notch signaling family of receptors plays a critical role in cell development, apoptosis and fate, therefore they have been considered as key targets for diagnosis and therapy in a myriad of diseases [5]. The role of Notch components in tumorigenesis has therefore been intensively studied in the oncogene or tumor suppressor context [6]. From the time when the Notch1 receptor was associated to UCC, a huge effort has been made to study the mechanisms and molecules associated with its dysregulation. A global perspective of the entire set of Notch signaling repertoire is still under progress for the discovery of molecular markers and/or therapy.

The objective of this study was to evaluate the protein expression level of these molecules to investigate the relevance in therapy and/or prognostic. The analysis consisted in protein expression quantification by using tissue microarray (TMA) and immunohistochemistry (IHC) platforms.

Materials and Methods

Tissue Samples

Study cases were selected from the archive file of the Department of Pathology, "Dr. Ignacio Morones Prieto" Central Hospital, in San Luis Potosi, Mexico. All cases were routine diagnostic surgical specimens including biopsies and cone excisions of the uterine cervix or hysterectomy sections. Tissues were fixed in buffered formalin, processed using standard procedures and embedded in paraffin. The collection included 205 patients diagnosed by two pathologists independently (C.O.O. and A.L.R.) according to World Health Organization classification criteria. Surgical specimens included 83 CIN1, 53 CIN2, 28 CIN3, and 23 malignant tumor cases. Eighteen samples of normal uterine cervix were included in the study.

Tissue Microarrays (TMAs)

H&E staining was first performed for each sample to select the area of interest on the block. A 1.5 mm diameter cylindrical core of the primary paraffin block was transferred to the composite paraffin blocks to construct TMA blocks by using the Quick-Ray Manual Tissue Microarrayer (IHC WORLD, LLC). After construction, the inverted TMA blocks were heated at 50 °C for 2 h. TMAs were sectioned at 5 μm thickness. Each TMA section was placed overnight at 60 °C and stained with H&E to evaluate tissue integrity [7].

Antibodies

Immunostaining was performed using mouse monoclonal anti-Cdc25A (1:50 dilution, Abcam ab2357), rabbit monoclonal anti-Notch1 (1:50 dilution, Abcam ab52627), rabbit polyclonal anti-Notch2 (1:800 dilution, Novus Biologicals NB600–879), rabbit polyclonal anti-Notch3 (1:100 dilution, Santa Cruz Biotechnology, Inc. sc-5593), rabbit polyclonal anti-Notch4 (3 μg/ml, Abcam ab33163), mouse monoclonal anti-HIC1 (20 μg/ml, Abcam ab55120), rabbit polyclonal anti-SEL1L (1:200 dilution, Abcam ab78298), rabbit polyclonal anti-ASCL3 (1:25 dilution, US Biological A3620), mouse monoclonal anti-DLL1 (25 μg/ml, US Biological D2727–75), rabbit polyclonal anti-MIB1 (1:50 dilution, US Biological M3753–01), goat polyclonal anti-BRD7 (1:50 dilution, Santa Cruz Biotechnology, Inc. sc-131,879), goat polyclonal anti-TLE (1:50 dilution, Santa Cruz Biotechnology, Inc. sc-13,373), mouse monoclonal anti-MTA1 (1:25 dilution, Santa Cruz Biotechnology, Inc. sc-17,773), goat polyclonal anti-CARP1 (CCAR1) (1:50 dilution, Santa Cruz Biotechnology, Inc. sc-69,263), goat polyclonal anti-DLEC1 (1:50 dilution, Santa Cruz Biotechnology, Inc. sc-99,867), rabbit monoclonal anti-Cytokeratin 13 (1:250 dilution, Abcam ab92551), mouse monoclonal anti-Cytokeratin 14 (1:150 dilution, Abcam ab7800), rabbit polyclonal anti-SOCS3 (1:100 dilution, Abcam ab16030), goat polyclonal anti-HES2 (1:50 dilution, Santa Cruz Biotechnology, Inc. sc-13,846), mouse monoclonal SNAI 1 (1:50 dilution, Santa Cruz Biotechnology, Inc. sc-271,977), mouse monoclonal anti-n-Myc (1:50 dilution, Abcam ab16898) and mouse monoclonal anti-CDKN2A/p16INK4a (1:200, Abcam ab54210).

Immunohistochemistry

Sections were deparaffinized and rehydrated in decreasing concentrations of ethanol, distilled water and PBS. When necessary, antigen retrieval was performed in a steam bath for 5–10 min at 90–95 °C in a citrate buffer (10 mM sodium citrate, 0.05% Tween 20, pH 6.0) or Tris-EDTA buffer (10 mM Tris-Base, 1 mM EDTA, 0.05% Tween 20, pH 9.0) [8]. The staining procedure was performed following the SuperPicture 3rd Gen IHC Detection Kit instructions (Invitrogen, Life Technologies) or SuperPicture Polymer Detection Kit (Invitrogen, Life Technologies) for mouse/rabbit or goat primary antibodies, respectively. Briefly, endogenous peroxidase activity was inhibited with peroxidase quenching solution (mouse or rabbit primary antibodies) or 3% hydrogen peroxide in absolute methanol (goat primary antibodies). TMA sections were incubated overnight at 4 °C with primary antibodies. When necessary secondary antibodies were used from Santa Cruz Biotechnology, Inc. including bovine anti-goat IgG-HRP (sc-2350), chicken anti-mouse IgG-HRP (sc-2954), and mouse anti-rabbit IgG-HRP (sc-2357). Samples were incubated with Polymer Conjugated HRP followed by DAB Chromogen and counterstained with hematoxylin.

Lastly, samples were dehydrated in ethanol, cleared in xylene, coverslipped and visualized in a Leica DM750 microscope.

Staining Quantification

Photographs from of each TMA core were obtained by using the LAS EZ software v2.0.0 for Windows (Leica Microsystem). Densitometric analyses of TIFF images were performed using the settings for Color Deconvolution plugins (http://www.mecourse.com/landinig/software/software.html) (v1.00r01) from the free Image-J software, NIH Image [9]. Briefly, color deconvolution was employed specifically in each selected area (ectocervical epithelium) for all cores to separate the channels of the DAB signal from the hematoxylin counterstaining. Data were reported as the mean intensity of pixels of the area measured as optical densitometric units (ODUs).

Statistical Analysis

Data analysis was performed using the R free software (version 3.1.1, http://www.r-project.org/) with a 95% confidence level. Normality and variance homogeneity were evaluated using the Shapiro-Wilk [10] and Levene procedures [11], respectively. Outliers were identified according to Tukey's definition and repetitions with outliers were eliminated. A power transformation was fitted by the BoxCox function [12] and the model was evaluated and compared to the reference model in order to find the smallest Akaike Information Criteria (AIC) model. Tukey's Honestly Significant Difference (HSD) procedure [13, 14] was applied to mean comparison. Lastly, the probabilities were calculated and adjusted using the Holm procedure [15] to achieve independence.

Results

Based on our mRNA study [4] we selected the top 15 upregulated molecules for protein evaluation: CCAR1/CARP1, CDC25, Notch3, MTA1, MYCN, Notch1, ASCL3, HIC1, SNAI1, SEL1, BRD7, E(sp1)/TLE, HES2, DLL1, and MIB1. Likewise, two downregulated molecules were included: DLEC1 and Notch4 [4]. In addition, we included commonly used biomarkers or key tumor molecules: p16/

Table 1 Statistical analysis for protein validation in TMAs

	Anova				HSD groups			
Protein	O	F	Padj	$AdjR^2$	a	b	c	d
SEL1	4	106.00	3.60E-15	0.717	NT	II	I, III, Tumor	–
Notch3	5	65.40	3.60E-15	0.621	NT	II, III, Tumor	I, III	–
SOCS3	1	61.00	3.60E-15	0.599	NT	II, III, Tumor	I, III	–
CARP1	2	57.30	3.60E-15	0.594	NT	Tumor	II, III	I, II
MIB1	1	59.80	3.60E-15	0.581	NT	II, III, Tumor	I	–
CK14	5	50.60	3.60E-15	0.571	NT	Tumor	II, III	I
CK13	21	49.40	3.60E-15	0.560	Tumor	NT, III	II	I
Notch2	8	45.60	3.60E-15	0.522	NT	II, III, Tumor	I	–
BRD7	11	41.40	3.60E-15	0.506	NT	I, Tumor	II, III, Tumor	–
ASCL3	5	30.70	3.60E-15	0.415	NT, I	II, III, Tumor	–	–
CDC25	1	25.20	3.60E-15	0.368	Tumor	NT, II, III	NT, I, II	–
DLL1	5	21.00	3.68E-13	0.323	NT, Tumor	I, II, III	–	–
Notch1	5	17.20	4.42E-11	0.275	NT	I, II, III, Tumor	–	–
MYCN	0	14.3	4.25E-08	0.265	II, III, NT, Tumor	I	–	–
HIC1	1	16.20	1.74E-10	0.264	NT	I	II, III, Tumor	–
Notch4	3	14.30	2.32E-09	0.247	NT, Tumor	III, Tumor	I, III	II, III
HES2	0	9.83	2.97E-06	0.214	II, III, NT, Tumor	I	–	–
DLEC1	3	10.30	6.63E-07	0.193	I, II	II, III	NT, III, Tumor	–
MTA	3	7.85	1.67E-05	0.142	I, Tumor	NT, II, Tumor	NT, II, III	–
p16	5	3.96	4.40E-03	0.0722	II, III, NT, Tumor	I, III, Tumor	–	–
SNAI1	0	3.82	5.45E-03	0.0646	II, III, NT, Tumor	I, II, III, NT	–	–
TLE	2	2.36	5.71E-02	–	–	–	–	–

O, Outliers; Anova F, F value; Padj, Adjusted probability (Holm, Hochberg); $AdjR^2$, Adjusted R-square

NT, normal tissue; I, CIN1; II, CIN2; III, CIN3

CDKN2A [16–19], cytokeratin (CK)13, CK14, Notch2, and SOCS3. The global analysis counted for a final study of 22 proteins (Table 1). To avoid visual bias, quantitative assessment of protein expression was performed using the free ImageJ software. This study was focused on the ectocervical area with the intention of detecting changes in the basal layer.

We found protein expression patterns similar to the transcriptional levels reported [4] especially those with increasing sustained expression. Table 1 summarizes the statistical data obtained for the HSD groups formed. Expression data can be observed in Fig. 1 for each chronological stage of the disease (ANOVA and number of groups with Tukey's HSD). As depicted, a number of highly expressed proteins (ANOVA, Padj $\leq 3.60E^{-15}$) were confirmed by this method (Table 1). The whole set of proteins was further analyzed and adjusted R-square (PadjR^2 = 0.717–0.599) showed a subset of proteins as highly expressed. Among them, SEL1L, Notch3, and SOCS3 showed a clear overexpression in CIN/tumor samples when compared to normal tissues (HSD groups, Fig. 1). Normal tissues exhibited consistent low basal protein levels against a strong reactivity observed in the development to tumor.

In order to validate the results, TMA cores were evaluated by two independent pathologists in blind routine sessions based on Klaes and Volgareva reports [20, 21]. The results with the staining categories are displayed in Table 2 and were defined as negative (0%), poor (<1%), sporadic (1–5%), focal (<25%) and diffuse >25%).

From the 40 normal specimens evaluated for SEL1L, 30 samples were negative (75%), 2 samples exhibited sporadic

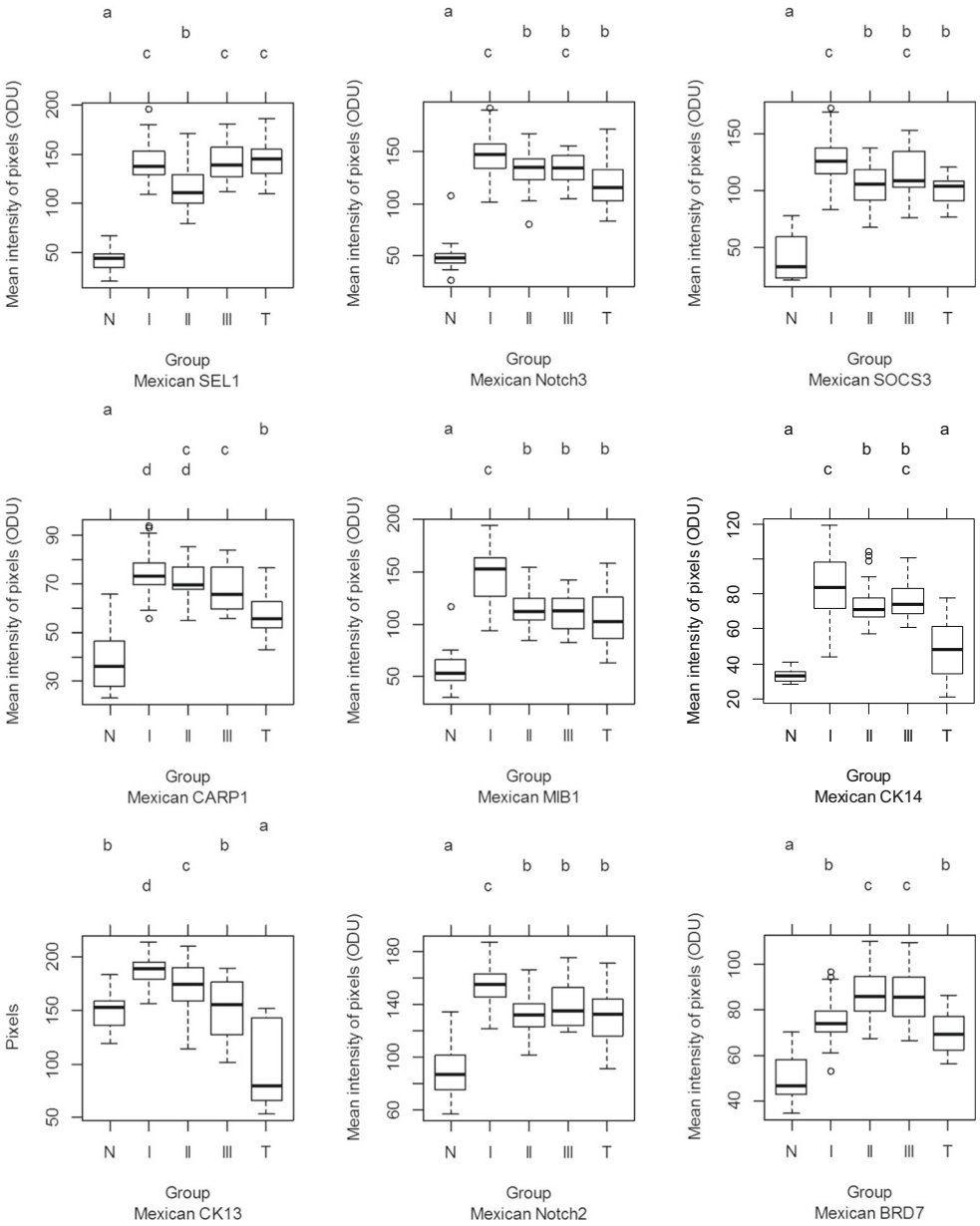


Fig. 1 Expression data of statistically significant proteins detected in the core specimens of TMAs in each chronological stage of the disease. ODU scores were obtained by using ImageJ. Groups formed based on HSD procedure are indicated at the top of each figure (a, b, c, or d). ODUs, optical densitometric units

Table 2 Protein expression staining in normal tissue, premalignant lesions and malignant tumors (blind evaluation by two independent pathologists)

Molecule and specimens evaluated	n	neg	poor	sporadic	focal	diffuse	ND
SEL1L							
Normal epithelium	40	30 (75)	0	2 (5)	7 (17.5)	1 (2.5)	0
CIN1	82	2 (2.4)	0	3 (3.6)	17 (20.7)	59 (71.9)	1
CIN2	53	0	0	3 (5.6)	43 (81.1)	7 (13.2)	0
CIN3 / cancer in situ	24	0	0	0	17 (70.8)	6 (25)	0
IC and SCC	23	0	0	0	3 (13)	20 (86)	0
Notch3							
Normal epithelium	40	26 (65)	4 (10)	7 (17.5)	0	3 (7.5)	0
CIN1	82	1 (1.2)	0	1 (1.2)	37 (45.1)	40 (48.8)	3
CIN2	53	0	0	2 (3.7)	40 (75.5)	11 (20.7)	0
CIN3 / cancer in situ	24	0	0	0	19 (79.1)	5 (20.8)	0
IC and SCC	23	0	0	0	9 (39.1)	14 (60.8)	0
SOCS3							
Normal epithelium	40	30 (75)	3 (7.5)	3 (7.5)	4 (10)	0	0
CIN1	82	1 (1.2)	0	6 (7.3)	42 (51.2)	28 (34.1)	5
CIN2	53	0	0	4 (7.5)	44 (83)	3 (5.6)	2
CIN3 / cancer in situ	24	0	0	0	17 (70.8)	3 (12.5)	4
IC and SCC	23	0	0	0	16 (69.5)	3 (13)	4

n, number of samples; neg, negative; IC, Invasive carcinomas; SCC, squamous cell carcinomas; ND, not detectable (core tissue lost). *Percentage is given in parenthesis

pattern (5%), 7 focal (17.5%), and one displayed a diffuse pattern (2.5%). The tendency for CIN1 cores was towards focal and diffuse pattern (20.7 and 71.9% respectively). In CIN2 specimens the main pattern found was focal (81.1%). CIN3 and cancer in situ presented mainly focal (70.8%), and tumors diffuse pattern was decisive (86%).

Notch3 analyses in normal samples were as follows: 65% of normal samples were negative. Four cores presented poor staining, seven sporadic staining and three were positive with diffuse pattern. The CIN1–3 specimens presented mainly focal and diffuse patterns, and tumors presented diffuse pattern.

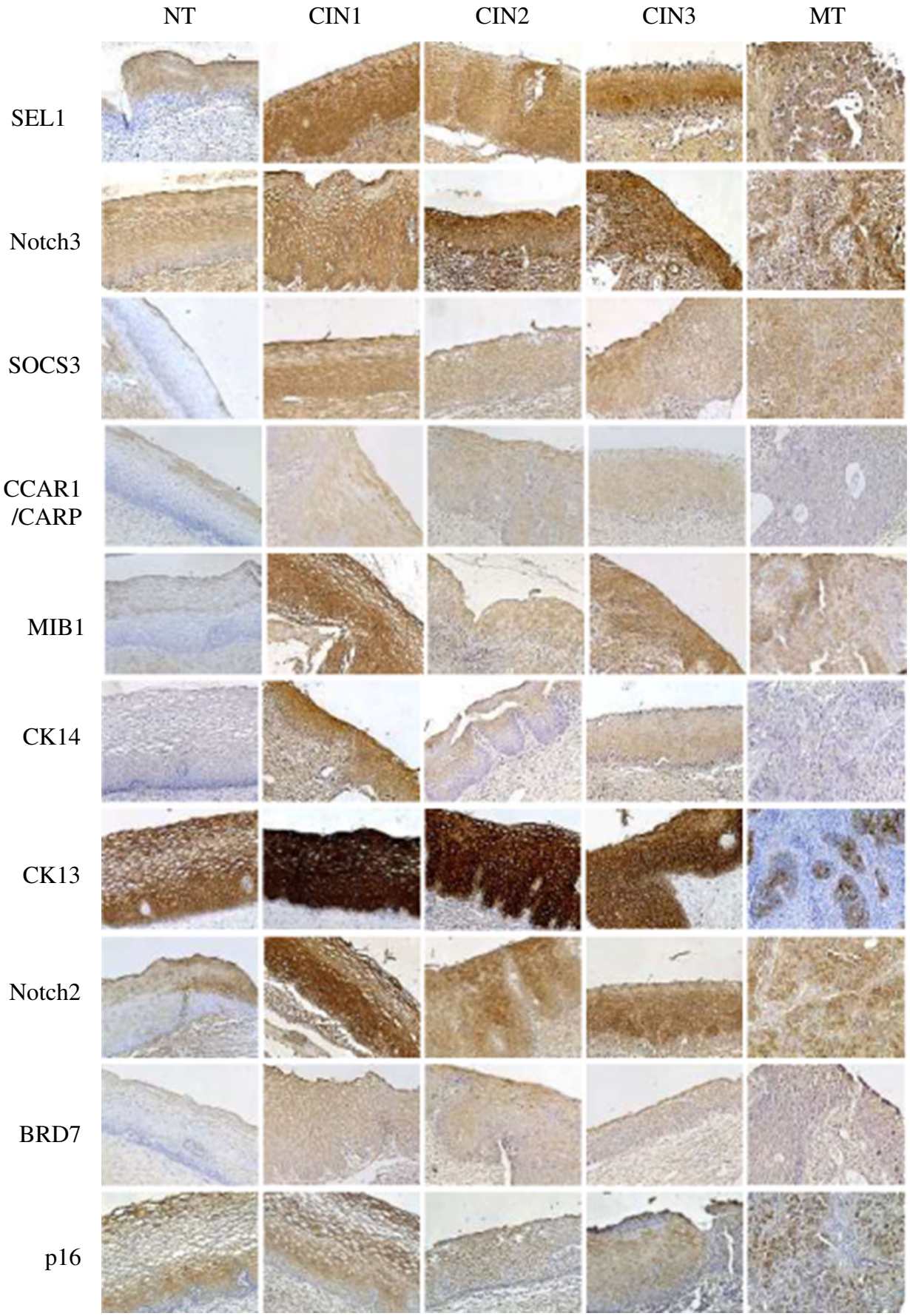
Likewise, SOCS3 presented the following expression pattern: from the normal epithelium, 75% were negative, 7.5% expressed poorly/sporadic and 10% presented focal staining. Lastly, CIN1–3 and tumors samples presented more than 50% of focal staining.

The data above analyzed are in agreement with the in silico quantitative approach; outliers were found and a tendency towards high expression levels along the progression stages (supplementary table).

Conclusions

SEL1L, an important component of endoplasmic reticulum-associated degradation (ERAD) pathway [22] was similarly expressed as in our previous report [4]. SEL1L (Suppressor/Enhancer of Lin-12-like) is an adaptor protein for the ubiquitin ligase Hrd1 in ERAD degradation and has been considered as a negative regulator of Notch proteins [23] that participates in several malignancies. SEL1L p38 variant has been detected in HeLa cells [24] but direct evidence has not yet been demonstrated in UCC tissues. SEL1L overexpression has been reported in colorectal cancer [25] and in neuroblastoma (NB); the role of destabilization of MYC/MYCN proto-oncogenes influencing the overexpression of SEL1L [26]. We found in our study that MYCN presented a stationary expression in normal and CIN/tumor stages. High expression/amplification of MYCN is frequently reported in diverse types of tumors, especially NB and glioma with poor prognosis [27]. Conversely, MYCN silencing produces apoptosis and differentiation in NB cells. Furthermore, inhibition is observed in pancreatic and breast adenocarcinomas [28]. It is probable that a similar destabilization mechanism or inhibition of MYCN could occur in UCC affecting SEL1L expression.

Notch3 overexpression was also confirmed. Recent reports confer on Notch3 a tumor suppressor function in distinct contexts [29–31]. Moreover, interactome analysis of Notch3 [32] provides additional evidence of its participation in ERAD events in ovarian cancer due to the presence of the PPxY motif. Upregulation of the Notch3 transcript was also documented in dexamethasone-induced SEL1L in breast cancer [33]. Our results are in accordance with Yeasmin [34] and Tripathi's reports [35]; a significant high expression was found in lesions against normal epithelium. It has been shown that Notch3 deregulation plays an important role in development of T-cell neoplasias; constitutive expression of the


NT
CIN1
CIN2
CIN3
MT
SEL1
Notch3
SOCS3
CCAR1
/CARP
MIB1
CK14
CK13
Notch2
BRD7
p16

◀ **Fig. 2** Immunostaining of the top ten differentially expressed proteins in TMAs from Mexican specimens. Photographs for representative core cases. Normal tissue (NT), cervical intraepithelial neoplasia (CIN) 1, CIN2, CIN3, and malignant tumor (MT). The scale bar represents 100 μM. Magnification is 10×

intracellular domain of Notch3 has been demonstrated in induced T-cell lymphomas in transgenic mice [36] whereas overexpression sustains T cell leukemogenesis [37]. These findings confirm the involvement of both Notch signaling molecules in our study model and their possible use in UCC as a complementary diagnostic marker.

The link between SEL1L and Notch3 is of great importance due their participation in protein quality control via proteasome. Any unbalanced expression of these molecules [38, 39] may represent a threat in the correct processing of protein degradation.

Lastly SOCS3, a member of the SOCS protein family has been described for its association to a vast number of signaling proteins and inhibition of selected cytokines. Sustained expression of SOCS3 has been observed in hematological malignancies as a probable tumor suppressor. Overexpression has been shown to reduce cellular proliferation and migration in prostate cancer [40, 41]. Recently, and in controversy with these reports, high SOCS3 expression was found in normal tissue against cervical cancer specimens [42], however, the apparent normal tissue was extracted from neighboring cervical cancer tissue, therefore, we cannot discard a probable context effect. Our study shows for the first time direct evidence of increased expression of SOCS3 in UCC with key statistical significance.

Control UCC markers CK13 and 14 were included in the study since they play a critical role in epithelium terminal differentiation. As expected, a sustained expression was found for CK13 when compared against CK14. A tendency of downregulation in the progression of the disease was observed for both proteins; however, the HSD groups overlapped between normal and tumor tissues (Figs. 1 and 2).

We did not find a clear statistical difference for the HPV-related p16 biomarker along the progression of the disease; although normal ectocervical epithelium displayed low reactivity, especially in the basal layer, only two HSD groups were formed (Table 1 and supplementary material).

We conclude that strong positivity of SEL1 and Notch3 with a clear switch from normal epithelium to CIN stages and cancer could be used to improve diagnostics in UCC in combination with p16. Combinatorial assessment of SEL1L/Notch3 or perhaps SOCS3 will be further studied to test the clinical and mechanistic utility in the Notch signaling pathway. Furthermore, in vitro studies at molecular and cellular level are currently under analysis for development of future therapeutic strategies in the context of UCC tumorigenesis.

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Compliance with Ethical Standards

Ethical Approval The bioethical committee of this Institution (Comisión de Seguridad e Higiene y Medio Ambiente en el Trabajo, CSHyMA-IPICYT) approved the experimental design of this work under the letter no. 003.

Informed Consent The manuscript does not contain any studies with human participants by any of the authors and archive cases remained anonymous.

Conflict of Interest The authors declare that they have no conflict of interest.

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