This is a pre-print of an article published in *Tumor Biology*. The final authenticated version is available online at:
https://doi.org/10.1007/s13277-010-0151-4

Identification of differential expression of transcripts throughout the progression of cervical cancer among Mexican patients

Leticia Santos[a] Ma. Fabiola León-Galván[a] Erika Nahomy Marino-Marmolejo[a] Ana Paulina Barba de la Rosa[a] Antonio De León-Rodríguez[a] Roberto F. González-Amaro[b] Ramón Gerardo Guevara-González[c]

[a]División de Biología Molecular, Instituto Potosino de Investigación Científica y Tecnológica A.C. (IPICYT), San Luis Potosí, México

[b]Departamento de Inmunología, Facultad de Medicina, UASLP, San Luis Potosí, México

[c]Facultad de Ingeniería, Universidad Autónoma de Querétaro, Centro Universitario, Querétaro, Querétaro, México

Key words: Cervical cancer, SSH, gene expression, CIN

Running head: Transcript profiling in Mexican patients with cervical cancer

Corresponding author: Prof. Dr. Leticia Santos

Camino a la Presa San José 2055, Col. Lomas 4a. sección,

78216 San Luis Potosi, SLP (Mexico)

Tel. +52 444 8342057, Fax +52 444 8342010.

E-mail lsantos@ipicyt.edu.mx

Abstract

Aim: To analyze the changes in the gene expression taking place during the chronological progression to cervical cancer in Mexican patients. **Methods:** This study was conducted comparing two CIN 1, four CIN 2, six CIN 3 and four microinvasive stage samples by suppression subtractive hybridization and Southern blotting. The validation of the results was done by Northern blot analysis. **Results:** Detection of Human Papilloma Virus 16 was detected in 10 of the lesions, other genotypes in two of the samples were found. After analyzing 1800 cDNA clones, we found 198 up-regulated, 166 down-regulated and no significant change of gene expression in 86 clones (p=0.005). The results were validated by Northern blot analysis (p=0.0001) in the identification of 28 over-expressed and seven down-regulated transcripts related to cancer and the Notch signaling pathway. **Conclusions:** We observed a directly involved set of genes that are essential for the transformation of cervical cells that sustain the development to malignancy and that could be used for the diagnostic and development of new therapeutic agents.

Introduction

Cervical cancer (CC) is the second most frequent cause of mortality among women worldwide [1-4] including Latin America [5-6]. The development of CC is defined through well-known steps: human papilloma virus (HPV) transmission, viral genomic integration, persistence, progression of gradually infected cells to neoplasia, and invasion [7]. The discovery of early diagnostic markers has been a central subject, an increasing number of data has been released to understand the molecular mechanisms that trigger the development and progression to CC through several platforms: oligonucleotide microarrays for the identification of expressed genes such as the silencing HPV RNA [8], complete transcriptome expression [9-10], and protein profiling [11-12]. The identification of molecules and linked pathways are a matter of extensive research at every stage of the disease [13-14] given the importance in the development of therapeutic agents such as antagonists of molecules in key cellular events [15-19].

The rationale of this work is to contribute to the understanding of the pathogenesis of CC through the analysis of the gene expression from biopsies taken at the different stages of the disease by the suppression subtractive hybridization approach (SSH). We report molecules linked to cancer and the Notch signaling pathway.

Materials and methods

Patients and tissue samples

The 16 cervical cancer samples (2 CIN 1, 4 CIN 2, 6 CIN 3 and 4 at microinvasive stage) were obtained from patients histopathologically diagnosed. The biopsies were snap-

frozen in liquid nitrogen and stored at -80°C. Morphologically normal cervical biopsies were taken from the same patients from a location distant from the lesions. All patients signed a letter of consent under the *Declaration of Helsinki* and the use of all samples and the experimental procedure for this study were reviewed and approved by the Ethics and Research Committee of the *Clinica de Displasias, Hospital General de Zona 01* at the *Instituto Mexicano de Seguro Social (IMSS)* in San Luis Potosi city, Mexico.

HPV analysis

Genomic DNA was extracted from all samples by using the Ultra Clean tissue DNA isolation kit (MO BIO laboratorios, Inc). The samples included in this study were subjected to PCR amplification using a set of primers targeting the E2, E6 and E7 oncogenes of the HPV-16 and the universal pair of primers MY 09-11 [20-21].

RNA isolation

The cervical cancer tissues and their corresponding normal tissues (tester and driver respectively) were subject to total RNA isolation with the RNeasy mini kit (Qiagen, Hilden, Germany). The quality, purity, and integrity of the extracted RNA were assessed by formaldehyde agarose gel electrophoresis (28/18S bands). RNA quantification was further determined spectrometrically by the $OD_{260/280}$ nm ratio.

Construction of the substracted cDNA library

One µg of total RNA of each condition (tester and driver) was used as template for the synthesis of the first strand of cDNA by using the Superscript II Reverse Transcriptase (Life Technologies, Rockville, MD, USA) and the SMART^{TM} PCR cDNA synthesis kit (Clontech, Palo Alto, CA, USA). The cDNA was amplified by long-distance polymerase

chain reaction (LD-PCR) at 15, 18, 21, 24, and 27 parallel cycles to compare and guarantee an optimal and suitable amount for the construction of the subtractive library. The amplification efficiency was monitored using placental RNA as positive control. CHROMA SPIN™ Columns (Clontech, Palo Alto, CA, USA) were used to guarantee highly pure cDNA. Suppression subtractive hybridization (SSH) was carried out using the PCR-Select^{TM} cDNA subtraction kit (Clontech, Palo Alto, CA, USA). The tester and driver cDNAs were digested using *Rsa* I to yield blunt ends. The tester cDNA fragments were divided into two aliquots and each one ligated in separated reactions with adapter 1 and adapter 2 resulting in two populations of tester cDNA. A small amount of each tester population were mixed with an excess of driver population (5 µg**)**, and subsequently heat-denatured, and allowed to anneal for 8 hours at 68°C. The two samples from the first hybridization were combined and annealed with additional fresh-denatured driver cDNA and annealed overnight at 68°C. A primary PCR was conducted to amplify those cDNAs that represented differentially expressed genes. A secondary PCR amplification was performed using nested primers 1- and 2R (Clontech, Palo Alto, CA, USA) to reduce background and for the preparation of Southern blot probes. The products of the secondary PCR amplification were monitored by agarose gel electrophoresis. In all cases, the efficiency of amplification was evaluated by comparing the expression levels of constitutive G3PDH gene.

Cloning, screening, and DNA sequencing of subtracted fragments

PCR fragments were ligated into the pCR4–TOPO cloning vector according to the manufacturer´s instructions (Invitrogen, Carlsbad, CA, USA). 2 µL of the ligation reaction were used to transform chemically competent *E. coli* TOP10 cells (Invitrogen, Carlsbad, CA, USA). The transformed cells were plated on LB agar containing kanamycin. Individual *E. coli* colonies were inoculated into LB media containing ampicillin/kanamycin and shaken

overnight at 37°C. Inserts were screened by restriction analysis using *Eco*RI (Invitrogen, Carlsbad, CA, USA) and agarose gel electrophoresis. 500 µL each selected colony of the library was stored at -80°C containing 50% glycerol.

Each clone generated was sequenced using an ABI PRISM 310 Genetic Analyzer (Perkin Elmer, Norwalk, CT, USA). Homology searches were performed using the NCBI BLAST program [22].

cDNA macroarrays by Southern blot analysis

0.2 µg of the insert-plasmids were spotted onto positive-charged nylon membrane (Hybond TM-N+, Amersham Biosciences, Buckinhamshire, UK) to construct 7x7 clone arrays by using the Slot manifold (Amersham Biosciences, Buckinhamshire, UK). Replicates of the SSH library were hybridized by Southern analysis either driver or tester cDNA probes. The probes were generated by incorporation of fluorescein-11-dUTP using the Gene Images™ Random Prime labeling kit according to the manufacturer's instructions (Amersham Pharmacia Biotech Inc, Piscataway, NJ, USA). Detection of fluorescein-labelled probes in Southern dot blots was performed with the anti-fluorescein alkaline phosphatase conjugate and CDP-Star detection reagent using the Gene Images CDP-Star detection module (Amersham Pharmacia Biotech Inc, Piscataway, NJ, USA).

Northern blot analysis

The mRNA isolates from each stage sample were pooled to minimize variability. 1.5 µg of the isolated mRNA was spotted into positive charged nylon membranes ((HybondTM-N+ Amersham Biosciences UK Limited) using the Slot manifold (Amersham Biosciences, Buckinhamshire, UK HP7 9NA). Probes were created from the previously isolated SSH fragments by incorporating fluorescein-11-dUTP (Gene Images CDP-Star random prime

labeling module) according to the manufacturer instructions (Amersham Pharmacia Biotech Inc, Piscataway, NJ, USA). The blots were hybridized, washed, and detected with the Gene Images CDP-Star detection module (Amersham Pharmacia Biotech Inc, Piscataway, NJ, USA) by using anti-fluorescein alkaline phosphatase conjugate and CDP-Star detection reagent and followed by three hours exposure. The controls consisted of the G3PDH gene.

Statistical analysis of expression data

Analysis of variance (ANOVA) was performed to evaluate significant differences (p=0.005) in expression levels of the lesions against non-lesions controls (Statgraphics 5.0 software, Manugistics, Inc.). The threshold mean difference in the level of expression was taken at 2.5-fold with respect to the controls. The confirmation of the results was validated by Northern blot analysis by decreasing the p value to 0.0001.

Results

HPV analysis

We analyzed the presence of HPV-16 in all the samples including the controls from each patient. From the total number (n=16), ten lesion samples presented HPV-16 (62.5%), five amplified positive for MY 09-11 (31.2%) and one classified as CIN 1, was HPV-negative (Table 1). Moreover, one apparently normal control from a microinvasive cancer patient amplified positive for the genes here analyzed thus we excluded that control for the SSH analysis.

Selection of differentially expressed genes

We used the SSH approach to identify the transcript population along the four transitional stages of the neoplastic transformation in cervical cells. The total RNA purified from the samples were quality evaluated spectrophotometrically by the 28S:18S ratio and integrity of the bands was visualized in agarose gels (Fig. 1a).

We obtained 1800 cDNA clones generated from two subtracted CIN 3 libraries and two subtracted microinvasive stage libraries. The inserts were amplified using the nested primers 1- and 2R, and the products were submitted to two rounds of amplification to ensure a representative amount of each enriched cDNA. Each product was fluorescein-labeled and used for the construction of the Southern blots macroarrays. Fluorescent-cDNAs were then hybridized against the cDNAs originally generated from each sample with the corresponding pair of negative controls. We analyzed the clones showing the most significant changes between negative controls and CIN 3/microinvasive cancer samples (p=0.005) in the first differential analysis. As a result of the Southern blot analysis we detected 198 clones with augmented expression, 166 clones with diminished expression and 86 clones without significant expression changes. These clones were examined by restriction analysis (Fig. 1b) and DNA-sequenced. The selected sequences were BLAST searched and annotated for their mRNA identity.

Nucleotide sequence accession numbers

The sequences that generated no similarity during the BLAST analysis were submitted to EMBL databank and assigned the following accession numbers: FN557216 (Homo sapiens EST, clone FFCC042), FN557217 (Homo sapiens EST, clone FFCC043), FN557218 (Homo sapiens EST, clone FFCC045), FN557219 (Homo sapiens EST, clone FFCC046), FN557220 (Homo sapiens EST, clone FFCC047), FN557221 (Homo sapiens

EST, clone FFCC048), FN557222 (Homo sapiens EST, clone FFCC050) and FN557223 (Homo sapiens EST, clone FFCC051).

Expression patterns of genes identified in each chronological neoplastic grade

The identified sequenced-clones were selected for their validation by Northern blotting. The study consisted of the hybridization of the fluorescence-labeled cDNA inserts selected from SSH and the original mRNA of the 16 paired-samples included in this study. To avoid bias in the overall expression among individuals, we pooled exact amounts of the mRNAs from each neoplastic stage (2 CIN 1, 4 CIN 2, 6 CIN 3 and 4 at invasive stage). The expression values of each group of genes were statistically analyzed (p=0.0001) in a two-class comparison, first to detect the genes presenting differential gene expression against the absolute values produced from the housekeeping gene G3PDH signal, and second to evaluate the non-neoplastic tissues (normal) in comparison to the neoplastic cervical lesions. The expression analysis consisted of the comparison of each band intensity. The normalization was based on the ratio between the gene/G3PDH absolute values followed by the ratio of the lesion/normal relative value expressed as fold change in figure 2 and table 2. The cutoff value to assign genes as increased/decreased expression was based on the relative value 1.0 produced by the normalized ratios.

We found a sustained expression throughout the progression of the disease in genes involved in the regulation of the cell cycle, development and metastasis (table 2). The fold changes comprehend 8 to 30 times of increasing and sustained expression when compared against the normal controls. Furthermore, we found a differential increasing expression during the progression of the disease in genes such as *NOTCH3*, *MTA1*, *v-myc*, *NOTCH1* and *ASCL3* in a range of 0 to 35 fold change. Likewise, a differential decreasing expression of the *NOTCH4* gene was found throughout the diverse stages. Other genes showing decreasing

expression, though with a less marked fold change; include *HHATL*, *DLEC1-S3*, *CDC2L2*, *CCNI*, and *MAPK12*.

Discussion

Cervical cancer remains a leading cause of death for women in Mexico [23]. The low impact of the Mexican National Cervical Cancer Screening Program has as a consequence the high incidence and mortality rates of late-stage cervical cancer. Consequently, we are interested in the screening and identification of potential markers that could be useful in the diagnosis as well as the study key mechanisms that trigger the development towards cancer.

We report here the generation and analysis of subtracted cDNAs corresponding to gene expression in each of the cervical neoplastic stages by the SSH method and Southern blot. We have confirmed the SSH library by showing that the genes selected for Northern blot validated the differential expression between each lesion grade. Our findings represent a collection of cancer-related genes and a variety of genes associated with cell development and cell-cycle mechanisms that have not been reported by other authors.

The identification of expression changes associated with stages of disease progression will help to understand new possible mechanisms in the development and progression of the disease. Our perspective is now to investigate the molecules reported at protein level.

Acknowledgments

We thank Mrs. Sydney Robertson Jiménez for the English correction of the manuscript.

References

1 Munoz N, Bosch FX, de Sanjose S, Shah KV: The role of HPV in the etiology of cervical cancer. Mutat Res 1994;305:293-301.

2 Bosch FX, de Sanjose S: Human papillomavirus in cervical cancer. Curr Oncol Rep 2002;4:175-183.

3 Bosch FX, de Sanjose S: The epidemiology of human papillomavirus infection and cervical cancer. Dis Markers 2007;23:213-227.

4 Bosch FX, Munoz N, Shah KV, Meheus A: Second International Workshop on the Epidemiology of Cervical Cancer and Human Papillomaviruses. Int J Cancer 1992;52:171-173.

5 Herrero R, Brinton LA, Reeves WC, Brenes MM, de Britton RC, Gaitan E, Tenorio F: Screening for cervical cancer in Latin America: a case-control study. Int J Epidemiol 1992;21:1050-1056.

6 Herrero R, Brinton LA, Reeves WC, Brenes MM, Tenorio F, de Britton RC, Gaitan E, Montalvan P, Garcia M, Rawls WE: Risk factors for invasive carcinoma of the uterine cervix in Latin America. Bull Pan Am Health Organ 1990;24:263-283.

7 Walboomers JM, Jacobs MV, Manos MM, Bosch FX, Kummer JA, Shah KV, Snijders PJ, Peto J, Meijer CJ, Munoz N: Human papillomavirus is a necessary cause of invasive cervical cancer worldwide. J Pathol 1999;189:12-19.

8 Min W, Wen-li M, Zhao-hui S, Ling L, Bao Z, Wen-ling Z: Microarray analysis identifies differentially expressed genes induced by human papillomavirus type 18 E6 silencing RNA. Int J Gynecol Cancer 2009;19:547-563.

9 Kim TJ, Choi JJ, Kim WY, Choi CH, Lee JW, Bae DS, Son DS, Kim J, Park BK, Ahn G, Cho EY, Kim BG: Gene expression profiling for the prediction of lymph node metastasis in patients with cervical cancer. Cancer Sci 2008;99:31-38.

10 Kendrick JE, Conner MG, Huh WK: Gene expression profiling of women with varying degrees of cervical intraepithelial neoplasia. J Low Genit Tract Dis 2007;11:25-28.

11 Wong YF, Cheung TH, Lo KW, Wang VW, Chan CS, Ng TB, Chung TK, Mok SC: Protein profiling of cervical cancer by protein-biochips: proteomic scoring to discriminate cervical cancer from normal cervix. Cancer Lett 2004;211:227-234.

12 Barba de la Rosa AP L-MO, Briones-Cerecero EP, Chagolla-López A, De León-Rodríguez A, Santos L, Vázquez-Ortiz G, Salcedo M.: Analysis of human serum from women affected by cervical lesions. J Exp Ther Oncol. 2008;7:65-72.

13 Gius D, Funk MC, Chuang EY, Feng S, Huettner PC, Nguyen L, Bradbury CM, Mishra M, Gao SP, Buttin BM, Cohn DE, Powell MA, Horowitz NS, Whitcomb BP, Rader JS: Profiling microdissected epithelium and stroma to model genomic signatures for cervical carcinogenesis accommodating for covariates. Cancer Research 2007;67:7113-7123.

14 Unger ER, Steinau M, Rajeevan MS, Swan D, Lee DR, Vernon SD: Molecular markers for early detection of cervical neoplasia. Disease Markers 2004;20:103-116.

15 Miele L: Rational targeting of Notch signaling in breast cancer. Expert Review of Anticancer Therapy 2008;8:1197-1201.

16 Rizzo P, Osipo C, Foreman K, Golde T, Osborne B, Miele L: Rational targeting of Notch signaling in cancer. Oncogene 2008;27:5124-5131.

17 Miele L, Miao H, Nickoloff BJ: NOTCH signaling as a novel cancer therapeutic target. Current Cancer Drug Targets 2006;6:313-323.

18 Miele L, Rizzo P, Osipo C, Foreman K, Bocchetta M, Tonetti D: Notch as a potential therapeutic target in cancer. Ejc Supplements 2008;6:4-4.

19 Nickoloff BJ, Osborne BA, Miele L: Notch signaling as a therapeutic target in cancer: a new approach to the development of cell fate modifying agents. Oncogene 2003;22:6598-6608.

20 Burd E: Human papillomavirus and cervical cancer. Clin Microbiol Rev. 2003;16:1-17.

21 Nees M, Geoghegan JM, Munson P, Prabhu V, Liu Y, Androphy E, Woodworth CD: Human papillomavirus type 16 E6 and E7 proteins inhibit differentiation-dependent expression of transforming growth factor-beta2 in cervical keratinocytes. Cancer Res 2000;60:4289-4298.

22 Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ: Basic local alignment search tool. J Mol Biol 1990;215:403-410.

23 Becker TM, Wheeler CM, Key CR, Samet JM: Cervical cancer incidence and mortality in New Mexico's Hispanics, American Indians, and non-Hispanic whites. West J Med 1992;156:376-379.

24 Cheng Q, Lau WM, Tay SK, Chew SH, Ho TH, Hui KM: Identification and characterization of genes involved in the carcinogenesis of human squamous cell cervical carcinoma. Int J Cancer 2002;98:419-426.

25 de Wilde J, Wilting SM, Meijer CJ, van de Wiel MA, Ylstra B, Snijders PJ, Steenbergen RD: Gene expression profiling to identify markers associated with deregulated hTERT in HPV-transformed keratinocytes and cervical cancer. Int J Cancer 2008;122:877-888.

26 Gulliksen A, Karlsen F: Microchips for the diagnosis of cervical cancer. Methods Mol Biol 2007;385:65-86.

27 Martin CM, Astbury K, McEvoy L, O'Toole S, Sheils O, O'Leary JJ: Gene expression profiling in cervical cancer: identification of novel markers for disease diagnosis and therapy. Methods Mol Biol 2009;511:333-359.

Tables

Table 1. Presence of HPV in the samples analyzed

Stage of progression	n=32	HPV-16 (E2, E6, E7)	Other HPV type (MY 09-11)	HPV negative
Controls*	16	1	2	13
CIN 1	2	1	0	1
CIN 2	4	2	2	0
CIN 3	6	4	2	0
Microinvasive cancer	4	3	1	0
Total	32	11	7	14

*Samples paired from the same patients from a distal location of the lesion

Table 2. Relative intensities in gene expression along the progression of CIN 1 to micronvasive cancer reported as fold change*

Gene symbol	CIN 1	CIN 2	CIN 3	Microinvasive cancer	NCBI Accession no.	Molecular function related
Increased gene expression						
CCAR1	31.44	31.68	35.75	33.74	NM_018237.2	Cell division cycle and apoptosis regulator
CDC25A	25.8	32.43	28.6	33.68	NM_001789.2	Rate-limiting oncogene
NOTCH3	1.01	13.73	16.00	32.84	NM_000435.2	Cell fate in development –Notch signaling pathway
MTA1	9.92	24.6	30.9	32.00	NM_004689.2	Metastasis
MYCN	6.87	6.87	30.4	31.57	NM_005378.4	*v-myc* myelocytomatosis viral related oncogene
NOTCH1	1.61	3.64	3.55	23.80	NM_017617.2	Cell fate in development –Notch signaling pathway
ASCL3	8.44	20.75	10.91	23.41	NM_020646.1	Cell fate in development
HIC1	19.26	27.9	20.46	20.00	NM_006497.2	Tumor suppressor
SNAI1	15.11	16.39	18.004	17.07	NM_005985.2	Metastasis
SEL1	11.01	17.40	16.62	16.77	NM_005065.3|	Cell fate in development –Notch signaling pathway
BRD7	14.09	14.91	14.91	15.45	NM_013263.2	Cell cycle progression
E(sp1)	14.09	14.37	15.95	14.88	NM_005078.1	Transcriptional repressor –Notch signaling pathway
HES2	11.01	15.46	17.23	14.00	NM_019089.3	Transcription regulator activity
DLL1	8.44	12.40	18.3	13.45	NM_005618.2	Cell fate in development –Notch signaling pathway
MIB1	7.37	11.58	11.58	11.98	NM_020774.2	Tumor marker
SPARCL1	9.30	13.0	12.6	11.93	NM_004684.3	Modulation of the structure of dermal extracellular matrix
MAML1	7.30	9.14	10.22	10.46	XM_001126853.1	Cell fate in development –Notch signaling pathway
SERPINA6	9.00	10.15	9.48	10.12	NM_001756.3	corticosteroid binding globulin precursor
GAS1	11.5	22.85	10.75	9.53	NM_002048.1	Growth suppressor / putative tumor suppressor gene
NUMB	13.04	16.55	12.36	9.53	NM_001005745.1	Cell fate in development
PIM3	7.75	8.68	8.17	8.79	NM_001001852.2	Oncogene
MBNL1	7.45	7.445	9.22	8.79	NM_207296.1	Tissue-specific alternative splicing regulators
SMARCA2	9.00	9.0	8.57	8.73	NM_003070.3	Regulation of transcription of certain genes by altering the chromatin structure
SART2	8.68	8.68	10.76	8.52	NM_013352.1	Tumor-rejection antigen
ADAM 7	7.41	9.33	7.29	8.03	NM_003817.1	Adhesion proteins and/or endopeptidases
PTTG1	15.62	17.4	15.65	7.63	NM_004219.2	Transforming and tumorigenic activities
PA2G4	12.47	12.47	12.64	6.80	NM_006191.1	Growth inhibition and induction of differentiation of cancer cells
TP53	2.03	2.03	1.96	1.94	NM_000546.2	Tumor suppressor
Decreased gene expression						
HHATL	0.933	0.956	0.98	0.84	NM_020707.2	Molecular function unknown - Notch signaling pathway
DLEC1-S3	0.593	0.59	0.59	0.616	NM_007337.2	Putative tumor suppressor
CDC2L2	0.588	0.56	0.561	0.578	NM_024011.2	Cell cycle control
CCNI	0.548	0.548	0.54	0.405	NM_006835.2	Regulators of CDK kinases
MAPK12	0.130	0.141	0.133	0.159	NM_002969.3	Signal transducer during differentiation of myoblasts to myotubes
NOTCH4	7.45	2.05	2.5	0.036	NM_004557.3	Cell fate in development –Notch signaling pathway

ANOVA p=0.0001, *Cutoff value =1.0

Figures

Fig. 1.

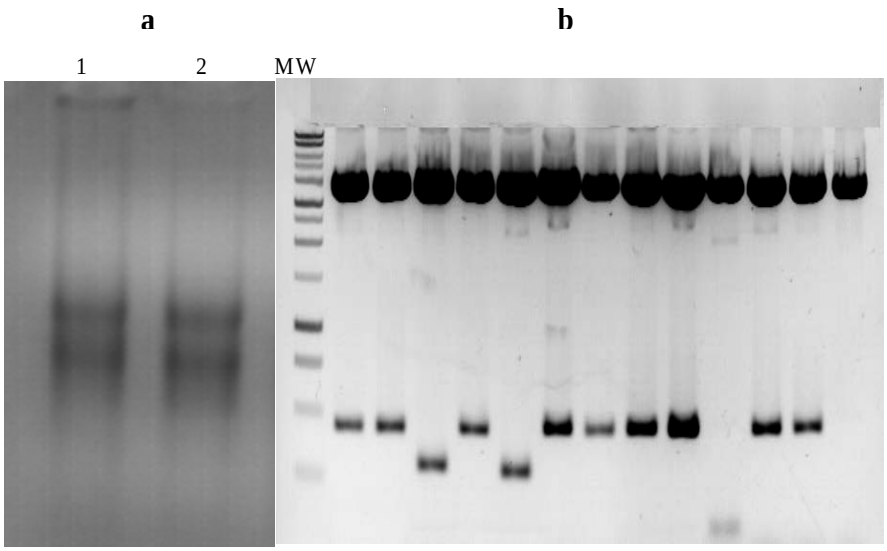

Fig. 2.

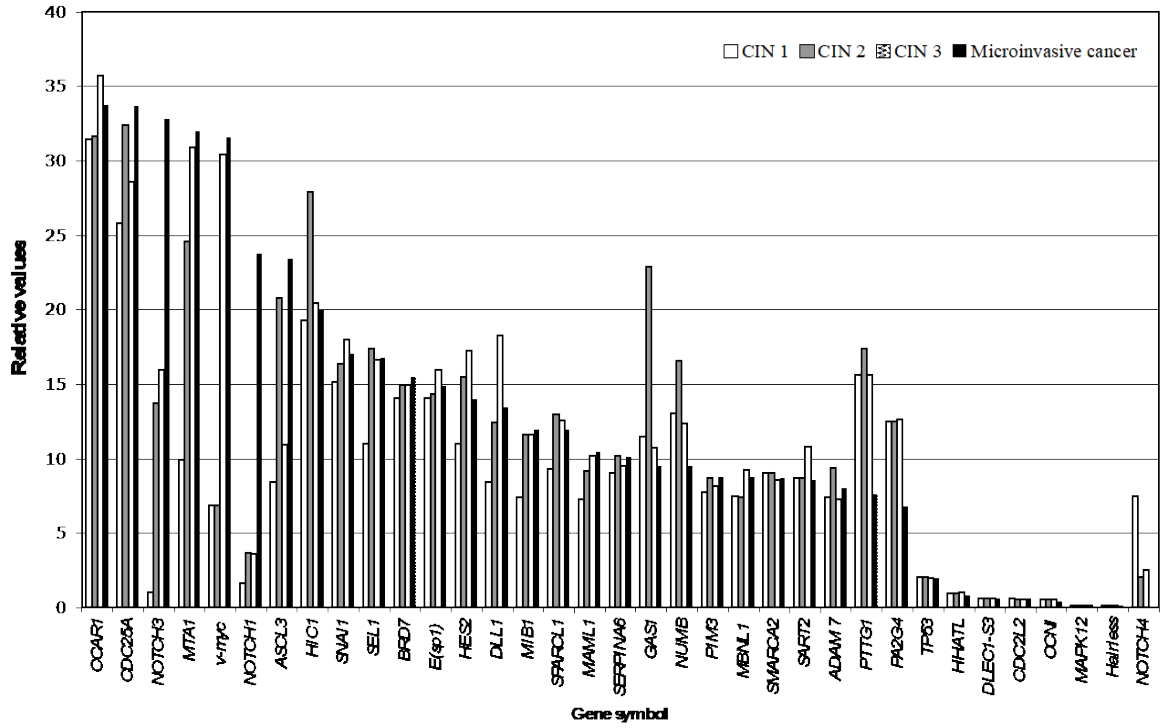
CIN 1
CIN 2
CIN 3
Microinvasive cancer
Relative values
0
5
10
15
20
25
30
35
40
CCAR1
CDC25A
NOTCH3
MTA1
v-myc
NOTCH1
ASCL3
HIC1
SNAI1
SEL1
BRD7
E(spl)
HES2
DLL1
MIB1
SPARCL1
MAML1
SERPINA6
GAS1
NUMB
PIM3
MBNL1
SMARCA2
SART2
ADAM 7
PTTG1
PA2G4
TP63
HHATL
DLEC1-S3
CDC2L2
CCNI
MAPK12
Hairless
NOTCH4
Gene symbol

Figure legends

Fig. 1. a Lane 1 and 2 are the visual assessment the 28S:18S rRNA ratio for the RNA quality of normal and lesion biopsies respectively. **b** Representative DNA digestions of the generated substractive libraries by *Eco*RI. MW, molecular weight marker in kbp (GeneRuler™ 1 kb, Fermentas).

Fig. 2. Gene expression profiles identified en each neoplastic stage. The expression values of each group of genes were statistically analyzed (p=0.0001) in a two-class comparison, first to detect the genes presenting differential gene expression against the absolute values produced from the housekeeping gene G3PDH signal, and second, to evaluate the non-neoplastic tissues (normal) versus the neoplastic cervical lesions.