Gynecologic Oncology 143 (2016) 406–413

Contents lists available at ScienceDirect

Gynecologic Oncology

journal homepage: www.elsevier.com/locate/ygyno

Comprehensive transcriptome analysis identifies pathways with therapeutic potential in locally advanced cervical cancer

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HIGHLIGHTS

- Genomic and transcriptomic analysis of patients with locally advanced cervical cancer
- The therapeutic potential of the JAK-STAT, NOTCH and mTOR signaling pathways in locally advanced cervical cancer
- Novel strategies should be considered in future clinical trials with LACC cancer patients to improve clinical outcomes

ARTICLE INFO

Article history:
Received 4 June 2016
Received in revised form 19 August 2016
Accepted 22 August 2016
Available online 28 August 2016

Keywords:
Locally advanced cervical cancer
Genomic and transcriptome analysis
Therapeutic targets

ABSTRACT

Objective. The objective of the present study was to provide genomic and transcriptomic information that may improve clinical outcomes for locally advanced cervical cancer (LACC) patients by searching for therapeutic targets or potential biomarkers through the analysis of significantly altered signaling pathways in LACC.

Methods. Microarray-based transcriptome profiling of 89 tumor samples from women with LACC was performed. Through Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis, significantly over-expressed genes in LACC were identified; these genes were validated by quantitative reverse transcription-polymerase chain reaction in an independent cohort, and the protein expression data were obtained from the Human Protein Atlas.

Results. A transcriptome analysis revealed 7530 significantly over-expressed genes in LACC samples. By KEGG analysis, we found 93 dysregulated signaling pathways, including the JAK-STAT, NOTCH and mTOR-autophagy pathways, which were significantly upregulated. We confirmed the overexpression of the relevant genes of each pathway, such as NOTCH1, JAK2, STAM1, SOS1, ADAM17, PSEN1, NCSTN, RPS6, STK11/LKB1 and MLTS8/GBL in LACC compared with normal cervical tissue epithelia.

Conclusions. Through comprehensive genomic and transcriptomic analyses, this work provides information regarding signaling pathways with promising therapeutic targets, suggesting novel target therapies to be considered in future clinical trials for LACC patients.

1. Introduction

Cervical cancer (CC) is the fourth most common cause of death in women worldwide, with approximately 527,600 new cases and 265,700 deaths in 2012 [1] In developing countries, such as in Latin America, sub-Saharan Africa and the Indian subcontinent, it is the second most common cause of cancer death in women as a result of more advanced disease at the time of diagnosis [2].

Persistent human papillomavirus (HPV) infection that has been linked to cervical carcinogenesis promotes profound changes in the transcriptional programs of epithelial cells, affecting complete signaling pathways [3]. The study of genomic information that allows us to analyze signaling pathways with promising therapeutic targets on CC deserves further and deep investigation. Recently, several targeted therapies have been developed to block specific signaling pathways, such as the JAK-STAT pathway (Ruxolitinib, Fedratinib and

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http://dx.doi.org/10.1016/j.ygyno.2016.08.327

tocilizumab), the Notch pathway (RO4929097, MK-0752, Anti-DLL4 mAb, OMP-21 M18 and OMP-59R) and the mTOR-autophagy pathway (sirolimus, temsirolimus, everolimus and ridaforolimus) [4–6]. These targeted therapies could improve the clinical outcome for up to 50% of patients with locally advanced cervical cancer (LACC) who failed initial treatment or those with recurrent disease [2,7].

The main goal of this study was to provide genomic information to improve clinical outcome in LACC patients by searching for therapeutic targets or potential biomarkers through the analysis of signaling pathways significantly altered in LACC. To achieve this goal, we analyzed the entire microarray-based transcriptome of 89 LACC tumor samples compared with normal cervix epithelia. Next, using bioinformatics tools to visualize the expression data in the context of pathway maps for cellular function (Kyoto Encyclopedia of Genes and Genomes, KEGG), we determined that the JAK-STAT, NOTCH and mTOR-autophagy signaling pathways were significantly upregulated. The expression levels of most of the over-expressed relevant genes were validated in an independent CCLA cohort and were compared with normal cervical tissues epithelia. Moreover, the protein expression was evaluated, and histopathological specimens were assessed to confirm the microarray results. These findings revealed new insights concerning LACC biology and the importance of upregulated signaling pathways that might be possibly blocked therapeutically.

2. Methods

2.1. Cervical samples

Cervical cancer tumors from 109 patients were obtained from 2010 to 2013 from the National Cancer Institute, Mexico City (INCan). All patients signed the informed consent form that was approved by the Ethical and Scientific committees of INCan (015/012/IBI-CEI/961/15). Immediately after surgical excision, the tumor biopsies were divided into two pieces: one for pathological confirmation and another for nucleic acid isolation.

Sixteen non-pathological cervical tissues were obtained from patients who had undergone a hysterectomy by uterine myomas. The inclusion criteria were as follows: a) no previous cervical surgery (such as the loop electrosurgical excision procedure or cone biopsy); b) no HPV infection; c) no hormonal treatment; and d) three previous negative Pap smears.

2.2. RNA purification and microarray hybridization

The cervical cancer transcriptome was obtained from eighty-nine LACC samples and 6 non-tumor tissues. RNA quality was assessed using the 18S:28S ratio. Hybridization targets and microarray preprocessing were performed as previously reported [8]. The microarray raw data are publicly available at the GEO database (Gene Expression Omnibus, http://www.ncbi.nlm.nih.gov/geo/) with the accession number GSE56303.

2.3. Validation of gene expression by real-time RT-PCR

Oligonucleotide primers for the target genes were designed based on the sequence data obtained from GenBank. The sequences of the primer sets used for RT-qPCR verification are listed in Supplemental Table 1. Beta-actin was chosen as an endogenous control reference. RT reactions were performed according to the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) using total RNA from 20 LACC samples and 10 normal cervix tissues. Real-time PCR was performed using Luminaris Color HiGreen qPCR Master Mix (Thermo Scientific) in StepOne Real-Time PCR System (Thermo Fisher Scientific), according to the manufacturer's protocol. Two replicates were run for each gene. The comparative Ct method (ΔΔCt) was used to quantify gene expression, and the relative quantification was calculated as $2^{-\Delta\Delta Ct}$ for the beta-actin housekeeping gene.

2.4. Validation of gene expression by immunochemistry

The expression of proteins in CC tissues implicated in the JAK-STAT, NOTCH and mTOR-autophagy and cellular pathways was obtained from the data deposited in the Human Protein Atlas [9]. The expression level of each protein was classified as low, medium or high relative to that of normal tissues.

2.5. Statistical analyses

To obtain a significant list of genes aberrantly expressed in tumor tissues in relation to normal counterparts, we employed significance analysis of microarrays (SAM) software, which identifies genes with significant changes in expression by assimilating a set of gene-specific t tests. For each gene, a score is assigned based on its change in gene expression relative to the standard deviation of repeated measurements for that gene. Genes with scores greater than the threshold are deemed potentially significant. We considered as positively or negatively regulated genes those with a delta score > 1.8 and less than − 1.8, respectively [10]. Retrieved genes were used to build a hierarchical cluster in which the heat maps represent differences and similarities based on the expression profiles (Fig. 1).

To identify the biological meaning of changes in gene expression, positively regulated genes in tumor samples examined by the SAM were submitted to the visualization tool Pathway Express (PE, a component of Onto-Tools suite). This widely used bioinformatics tool allows for the visualization of expression data in the context of KEGG biological pathways. The importance of PE is that it generates an impact factor (IF) of the entire pathway involved and a perturbation factor for each gene involved in a specific signaling pathway, thus providing a clearer representation of the alteration level for each biological pathway [11].

All data are expressed as the mean ± S.D. from two independent experiments. Statistical analyses were performed using Student's *t*-test. $P < 0.05$ (*) or $P < 0.01$ (**) was considered to indicate significance.

3. Results

3.1. Patients

One hundred nine LACC patients were enrolled: LACC samples from 89 patients were subjected to hybridization microarray, and 20 were used to validate the gene expression profile obtained from the microarray assay. The median age of the patients was 48 years (range, 29–69 years). Most patients had been diagnosed with squamous cell carcinoma (90.8%), and were in stage IIB (60.5%) or IIIB (24.7%) at the time of diagnosis (Table 1).

3.2. Gene expression profile based on cluster analysis

To identify the differential gene expression profile of LACC samples compared with non-pathological cervical epithelia, we used the algorithm SAM (http://www.stat.stanford.edu/~tibs/SAM), which identifies genes with significant changes in expression using the cut-off values of a delta score (score(d) ≥ 1.8 and ≤ 1.8 with a false discovery rate (FDR) < 10%).

Thus, we obtained a list of 13,065 genes (7530 up- and 5535 down-regulated) significantly altered in tumor samples versus their normal counterparts. Hence, a hierarchical cluster was built using Genesis software (Fig. 1). In general, the tumor samples showed a higher grade of homology among them than the normal samples, which had a more heterogeneous gene expression profile. This observation suggests that, although HPV infection is associated with several changes in the host

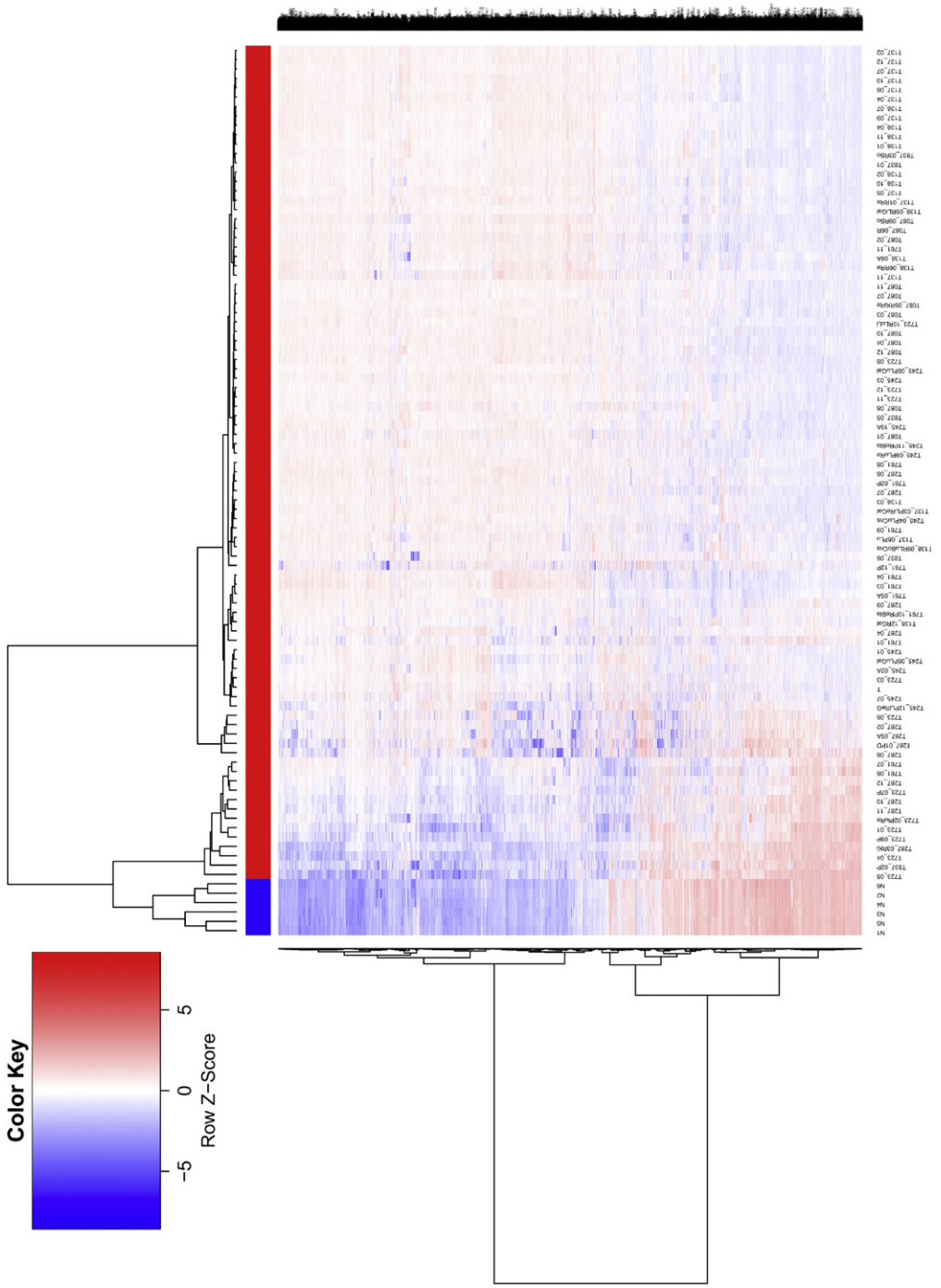

Fig. 1. Hierarchical cluster generated from 89 LACC and 6 non-tumor tissue samples. Cluster analysis of the microarray data. The microarray data were analyzed by the Genesis program [32]. The cluster shows 13,065 genes (7530 up- and 5535 down-regulated). Each row represents a gene, whereas each column corresponds to a tissue sample, the color line above the tissue samples indicates the sample type: a) normal samples (blue) and b) tumor samples (red). The relative abundance of each gene in the tissue correlates with the color intensity (red, induced; blue, repressed; white, no change). On the dendrogram, all six normal cervical samples clustered together, indicating their similarity based on the expression profile.

Table 1
Clinical characteristics of all patients.

Characteristic	All patients (109) N (%)
Age	48
Range	29–69
Tumor stage (FIGO)	
IB2	14 (12.0)
IIA	1 (0.91)
IIB	66 (60.5)
IIIA	1(0.91)
IIIB	27 (24.7)
Histology	
Squamous cell carcinoma	99 (90.8)
Adenocarcinoma	10 (9.1)
Tumor size	
≤4 cm	61 (55.04)
≥4 cm	48 (44.03)

cell, in the late stages of the carcinogenesis process, the pattern of gene expression induced by carcinogenesis process is similar.

3.3. Signaling pathway analysis

One of our main goals was to identify relevant cellular pathways aberrantly expressed between tumor and normal tissues, whose pharmacological inhibition as adjuvant therapy for LACC appears reasonable based on available evidence. To that end, we used PE, which is a component of Onto-Tools used to visualize expression data in the context of the KEGG Biological Pathway. PE retrieves an IF that can help to obtain a parameter of the alteration level in each cellular pathway. Thus, we imported a list of 7530 genes significantly over-expressed in LACC samples compared with normal tissues into the program to convert the expression data into pathway illustrations. Thus, 93 IF top-rated signaling pathways were obtained in tumor samples (Supplemental Table 2). The most affected cellular pathways were cell adhesion molecules (CAMs) and adherens junctions, which are involved in maintaining tissue architecture and cell polarity. Both pathways are related to metastatic processes, a finding that is expected because the clinical samples employed in this study were obtained from invasive tumors. Nonetheless, of the 93 cellular pathways, we focused on those that offer an important therapeutic potential such as the JAK-STAT, NOTCH and mTOR-autophagy signaling pathways.

An important aspect of PE is that it calculates a perturbation factor or perturbation index. The perturbation factor considers (i) the normalized fold change of the gene and (ii) the number and amount of perturbation genes downstream from it. The gene perturbation factor reflects the relative importance of each differentially regulated gene [11]. In this context, the JAK-STAT signaling pathway had sixty genes from 156 with a perturbation index from 0.7 to 9.3. JAK2 (Janus Kinase), STAM1 (Signal transducing adaptor molecules) and SOS1 (Son of sevenless homolog 1) were selected for validation experiments by RT-qPCR and protein expression. Regarding the NOTCH signaling pathway, 32 genes of 48 were altered, with the perturbation index ranging from 0.4 to 2.0. NOTCH1 (Notch homolog 1, translocation-associated), ADAM17 (ADAM metallopeptidase domain 17), PSEN1 (presenilin-1) and NCSTN (nicastrin) were selected for validation experiments by RT-qPCR and protein expression (Fig. 2). Regarding the mTOR-autophagy signaling pathway, 32 of 52 genes were found to be deregulated with a perturbation index ranging from 1.3 to 12.5. Hence, we chose RPS6 (ribosomal protein S6), STK11/LKB1 (serine/threonine kinase 11) and MLTS8/GBL (MTOR Associated Protein, LST8 Homolog) as validation targets (Fig. 3).

3.4. Experimental validation of deregulated genes in CCLA tumor specimens

To validate the experimental data, we analyzed the expression levels of individual genes of the JAK-STAT signaling pathway and the mTOR-autophagy signaling pathway. Hence, using RT-qPCR, the expression levels of NOTCH1, JAK2, STAM1, SOS1, ADAM17, PSEN1, NCSTN, RPS6, STK11/LKB1 and MLTS8/GBL were evaluated in an independent cohort consisting of 10 normal tissue cervix and 20 tumor tissues. As shown in Fig. 4, all of the genes were over-expressed significantly ($P > 0.0001$). These results were consistent with the results obtained from microarray analysis.

3.5. Immunohistochemical assessment of overexpressed genes with therapeutic potential

To further confirm the gene expression levels obtained by microarray experiments, we used immunohistochemical analysis to analyze the expression of ten differentially expressed genes in tumors and their normal counterparts. Therefore, we accessed the Human Protein Atlas database (http://www.proteinatlas.org/), which has information on protein expression in tumor and normal specimens. In agreement with the microarray analysis data, positive immunostaining in tumor samples was observed for JAK2 (10 positives from 12 analyzed tumor tissues), STAM1 (11 positives from 12), SOS1 (9 positives from 12), and NCSTN (9 positives from 10), and all of the tumor tissues exhibited positivity for ADAM17, NOTCH, STK11/LKB1, PSEN1, RP61 and MLTS8/GBL. It is worth noting that high- and/or medium-level expression was present at a rate of ~75% in tumoral cells (Fig. 5). Most of these proteins were localized in the cytoplasm and membrane except for the SOS1, PER2 and STK11/LKB1 proteins, which were located in the nucleus. Thus, 91.7% of the samples were pathologically classified as squamous cell carcinomas, and the remainder (8.3%) was classified as adenocarcinomas (Fig. 5).

4. Discussion

Despite early screening programs for cancer detection and vaccines, the impact on disease control has been limited for LACC diagnosed patients [12]. Concurrent chemo-radiotherapy followed by radical surgery are the principal treatments for LACC; however, approximately 30–50% of patients develop recurrent disease, which is a major cause of death [2–7]. Moreover, some of these patients are not candidates to receive cisplatin due to different co-morbidities [13]. In this respect, new target therapies such as Ruxolitinib, Fedratinib and tocilizumab (JAK-STAT pathway inhibitors), RO4929097, MK-0752, Anti-DLL4 mAb, OMP-21M18 and OMP-59R (Notch pathway inhibitors), sirolimus, temsirolimus, everolimus and ridaforolimus (mTOR-autophagy pathway inhibitors) have been developed and they can be promising, but further studies needed in LACC.

In an effort to provide genomic information with a special focus on searching for promising therapeutic targets that could improve the clinical outcome in LACC patients, we conducted a transcriptome analysis on 89 LACC tumor samples because our main was to show differences between normal tissues and cancer samples, obtaining 7530 genes that were significantly over-expressed. Next, gene annotation in biologically relevant databases resulted in 93 dysregulated signaling pathways (Supplemental Table 2). JAK-STAT, NOTCH, and mTOR-autophagy pathways represents the most important pathways involved in cell survival, differentiation, cell growth, progression and metastasis, and moreover can be pharmacologically blocked. The data generated by The Cancer Genome Atlas were used to analyze druggable cancer driver genes and were identified as those linked to the Wnt, Notch, JAK/STAT, NF-κB and MAPK signaling pathways, but, unfortunately, the data of CC have been removed [14]. In this sense, JAK-STAT and NOTCH pathways recently were identified by our research group as key pathways affected in CC [15]. There is no information about the role of JAK-STAT pathway

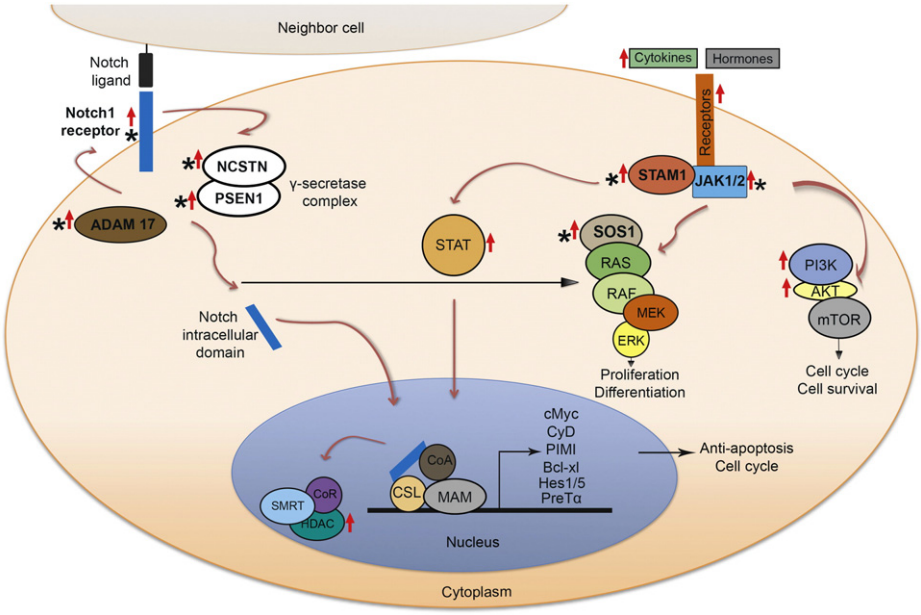

Fig. 2. Schematic representation of JAK-STAT and NOTCH cellular pathways altered in locally cervical cancer patients. Some genes implicated in Notch signaling are well known to play significant roles in carcinogenesis and tumor progression. Cytokines and hormones bind to receptors to activate phosphorylation and SOS1-Ras-Raf-Erk signaling to induce proliferation and differentiation. STAT molecules are activated and translocate to the nucleus where they bind to the promoter regions of target genes involved in anti-apoptosis and the cell cycle. In addition, cells transmit the signal through ligands binding to them at the NOTCH receptors [1–4], which are cleaved by a member of the ADAM family (ADAM17 and ADAM10) and gamma-secretase complex (PSEN1/2, NCSTN), leading to the release and nuclear transportation of NOTCH intracellular domain (NID), which regulates target gene transcription [5]. The red arrows indicated higher gene expression, and the stars indicate genes validated by qPCR in LACC samples.

in cervical carcinogenesis or clinical association; we consider significant to focus on these poorly characterized pathways in order to increase the current knowledge about them. Respecting to Notch pathway, despite of some reports showing its role in cervical carcinogenesis [16], there is no information about the importance of this pathway as a therapeutic target in locally advanced cervical cancer (LACC) patients. In this sense, we describe new molecular pathways susceptible to be used as therapeutic targets in the treatment of patients with cervical cancer. Additionally, the mTOR-related pathways represent the most important pathways involved in progression, invasiveness and metastasis, and

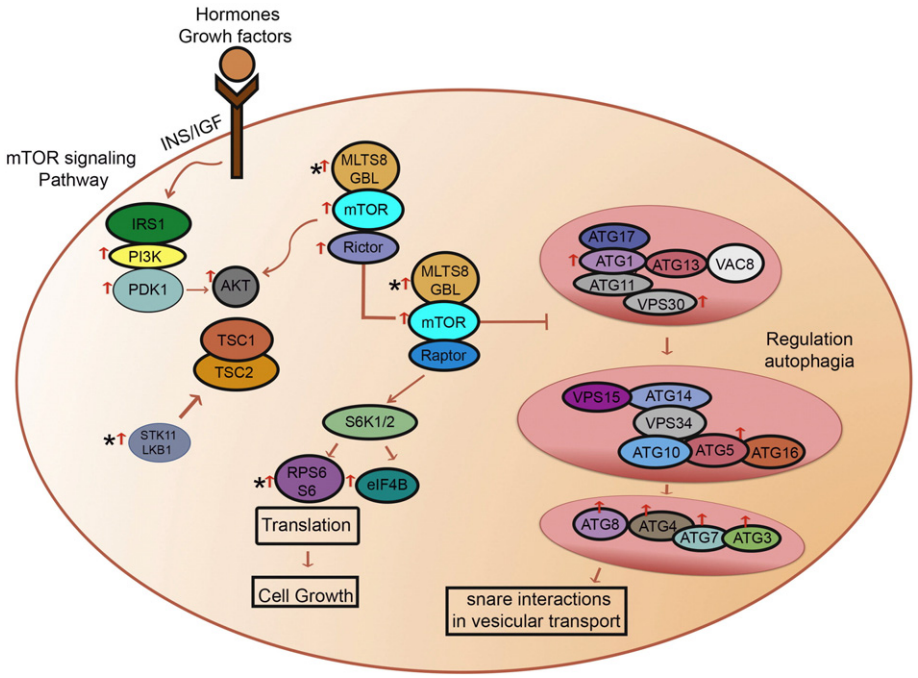

Fig. 3. Schematic representation of the mTOR-autophagy cellular pathways altered in locally cervical cancer patients. mTOR-autophagy signaling is implicated in cancer development and progression, and many of its components are amplified in human cancers. Insulin, hormones and growth factors activate the PI3K/Akt signaling cascade. Akt phosphorylates and destabilizes the TSC1/2 complex, promoting mTOR activation. The complex mTOR, MLTS8/GBL and Raptor phosphorylates S6 (RPS6) to modulate cell growth. Downstream targets of the complex mTOR, MLTS8/GBL and Rictor are AKT and PKC, which regulate cell survival. In contrast, mTOR negatively regulates autophagy by inhibiting Atgs such as Atg13 and Atg1 (ULK1/2). STK11/LKB1 modulates AMPK activity [6]. The red arrows indicate higher gene expression, and the stars indicate genes validated by qPCR in LACC samples.

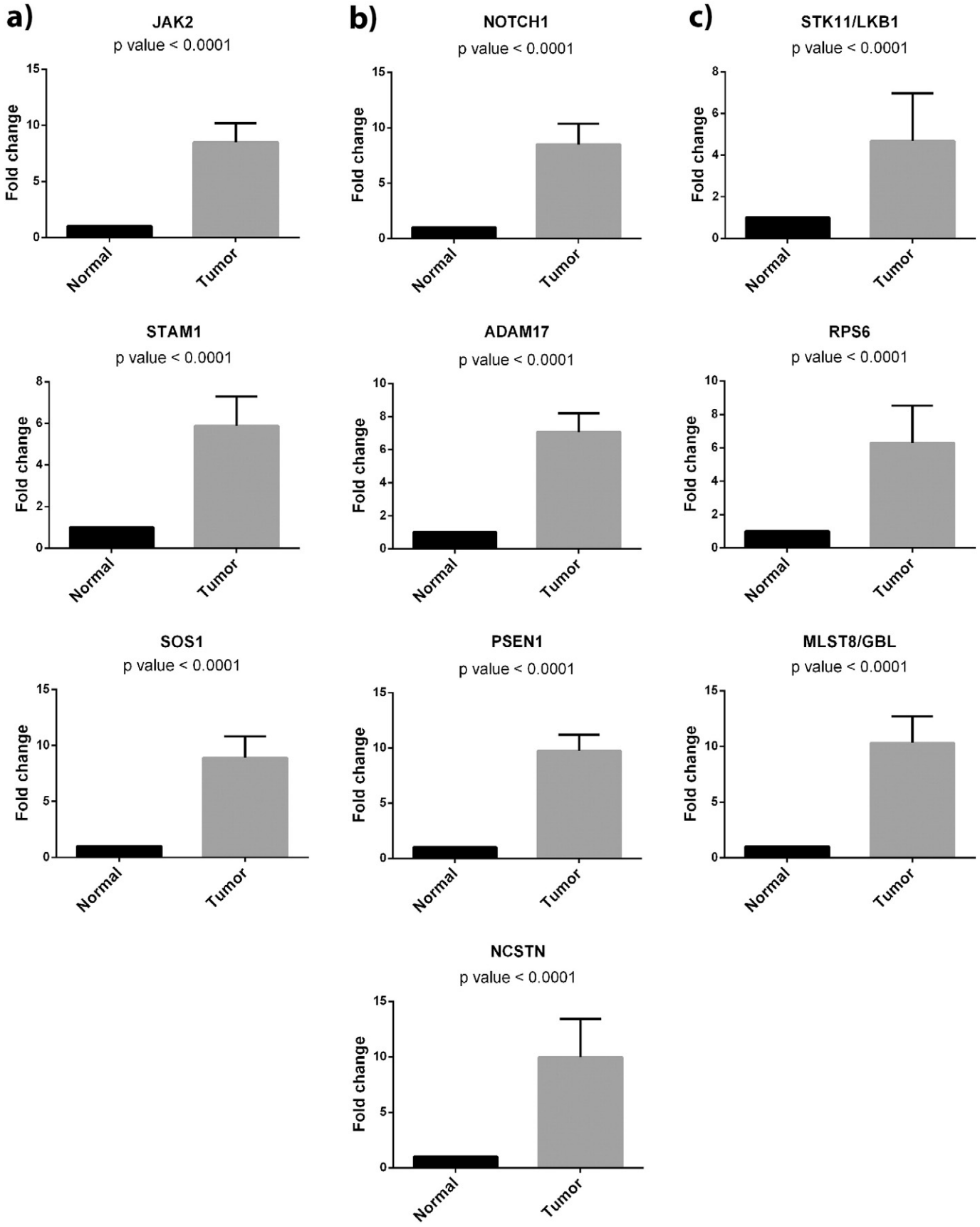

Fig. 4. Analysis of the relative expression of dysregulated genes in LACC and normal cervix tissue. The expression patterns of genes by RT-qPCR were determined as described in the Materials and Methods section. Statistical analysis to compare the mRNA expression levels between normal and tumor tissues was performed using an unpaired two-tailed *t*-test. A) Representative genes from the JAK-STAT signaling pathway. B) Representative genes from the NOTCH signaling pathway. C) Representative genes from the mTOR signaling pathway.

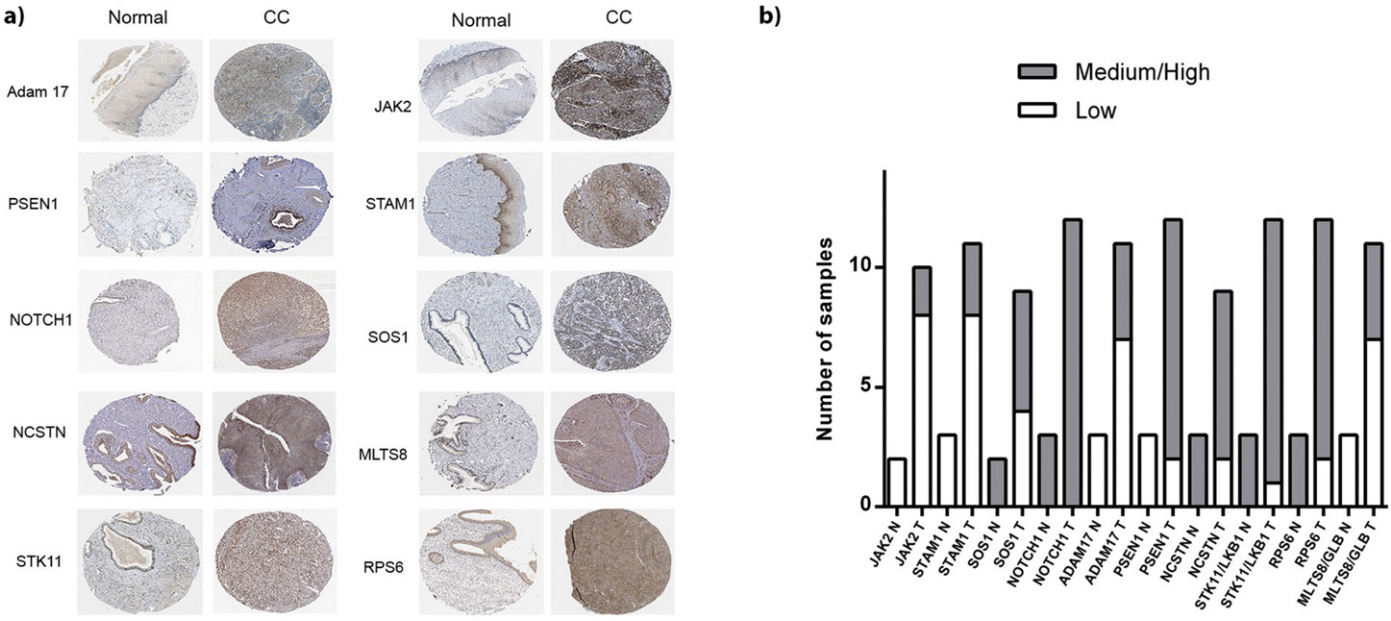

Fig. 5. Immunohistochemical analysis of overexpressed proteins in LACC. A) The data were collected from the Human Protein Atlas for normal and cervical carcinoma tissues. All images of immunohistochemistry-stained cancer tissues are available as high-resolution images in the cancer tissue atlas. B) Graphical representation of the number of samples analyzed according to the level of expression. T (Tumor Tissue) and N (Normal tissue).

moreover can be pharmacologically blocked, nevertheless, in LACC their clinical relevance is limited whereby their study can provide new and important clinical information to patients with advanced disease.

In our findings, we have shown the consistent overexpression of key molecules of the JAK-STAT, NOTCH and mTOR-autophagy pathways by microarray, qRT-PCR and immunohistochemistry in LACC. To our knowledge, this is the first study where the messenger RNA expression of SOS1, STAM1, MLST8/GBL and gamma-secretase complex (PSEN1 and NSCTN) was analyzed in CC.

Previously, it has been reported in prostate carcinomas and advanced ovarian cancer that SOS1 overexpression correlates with cancer progression and with a shorter survival, indicating that its dysregulation is an essential requirement for tumorigenesis [17]. We noticed that MLTS8/GBL mRNA and protein were overexpressed in CC, its expression has been evaluated in other tumors, such as prostate and colon cancer, and contributes to tumor growth and invasion; in addition, strong protein expression has been observed in invasive cancer lesions [18].

RPS6/p70S6K is a key protein of mTOR signaling (Fig. 3), which has been reported to be increased in cervical squamous cell carcinomas, and the overexpression was significantly associated with the occurrence of distant metastasis, similar to our results [19]. Temsirolimus, everolimus and ridaforolimus are mTOR inhibitors that have been evaluated for phase II trials in patients with advanced or recurrent gynecological cancer, temsirolimus has been shown a modest clinical benefit in 37 metastatic LACC patients, most patients experienced stable disease, suggesting that patient preselection with prognosis biomarkers would be very useful [20]. As expected, we found that the expression level of messenger RNA and protein from STK11/LKB1, RPS6 and MLTS8/GBL was upregulated, due to the overexpression of mTOR proteins that is associated with an increasingly aggressive clinical course and that has been reported to be useful for targeted therapy [21]. Unexpectedly, the tumor suppressor gene STK11/LKB1 was over-expressed, but our findings are consistent with those of a previous report showing that STK11/LKB1 expression is higher in cervical tumors than in normal tissues [22]. Moreover, LKB1 is mutated in at least 2% of cervical tumors; therefore, over-expression of the gene could be a mechanism to attenuate the tumor phenotype [23]. Similarly, stronger LKB1 expression in colorectal carcinomas than in normal mucosa has been reported [24].

Similar to our results, JAK2 overexpression has been reported in breast infiltrating ductal carcinoma, an aggressive and rare tumor type with a poor prognosis, and was associated with the mechanism of resistance after neoadjuvant chemotherapy [25]. JAK2 inhibitors such as Ruxolitinib, fedratinid have been shown that are well-tolerated and they improve overall survival in advanced pancreatic adenocarcinoma, advanced ovarian cancer and myelofibrosis patients [26].

Concordant with our results, has been reported that NOTCH1 and ADAM17 expression is significantly higher in CC than in normal tissues; these proteins have been significantly associated with tumor differentiation, aggressive progression and poor prognosis, suggesting that NOTCH signaling may be involved in CC progression [26–27]. The use of the NCSTN antibody in breast cancer cells decreased NOTCH1 expression and reduced the invasive capacity of the cells [27]. In addition, gamma secretase (PSEN and NSCTN) inhibitors have been shown to have the potential to sensitize breast cancer cells to chemotherapeutics that block chemoresistance [28,29]. In CC patients, treatment with RO4929097 (gamma secretase inhibitor) plus capecitabine to overcome chemotherapy resistance exhibited a clinical benefit and tolerance to side effects [30,31].

This work provides information regarding signaling pathways with promising therapeutic targets, with these findings, it is reasonable to speculate that the overexpression of key molecules of the JAK-STAT, NOTCH and mTOR pathways make them potential molecular therapeutic targets, although a limitation of our work was to validate the microarray results in only 20 LACC samples, these will need to improve further in a large set of LACC samples.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.ygyno.2016.08.327.

Conflict of interest statement

All authors declare that there are no conflicts of interest.

Acknowledgments

Supported by the Council for Science and Technology (CONACyT) (SALUD-2015-1-262044 and SALUD-2014-1-233733).

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