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# 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

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## 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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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

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