

Original Article

Interferon epsilon mRNA expression could represent a potential molecular marker in cervical cancer

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Abstract: The effects of the immune system response in the malignant transformation process have been described. Molecules such as interferons are involved in such process. Interferons are small single-chained glycoproteins, involved in the first line of defense against pathogens such as viruses, bacteria, and parasites. Interferon epsilon (IFN ϵ) is located in the 9p21.3 cytogenetic region, transcribes into a single exon mRNA. Contrary to other family members, IFN ϵ exerts low antiviral activity. In the present work molecular alterations such as copy number variation (CNV) and expression were analyzed by available microarrays and fifty-nine cervical tissues ranging from normal to cancer and three cell lines were assessed for IFN ϵ expression by RT-PCR, immunohistochemistry, and immunocytofluorescence. No significant CNV alterations were observed. Positive immunosignal was primarily present in the proliferative basal strata cells in the normal tissue, whereas in cervical cancer, all epithelial transformed cells were positive. The cell lines analyzed were HPV16, -18, and negative, all three cell-lines were positive for cytoplasmic protein presence. Interestingly, at the mRNA level, increased band intensity was observed, as the lesions were higher, and IFN ϵ up-regulation in CC ($P=0.0001$) is reported here. Our results suggest that up-regulation is present as an independent event from single or multiple HPV infection ($P=0.90$). In conclusion, we suggest that IFN ϵ mRNA up-regulation could represent a potential molecular marker in CC. Expression of IFN ϵ might not be related to HPV infection or CNV, which could have an important role in cellular homeostasis and could influence immune related events in cervical carcinogenesis.

Keywords: Cervical cancer, cytokines, interferon epsilon, molecular marker

Introduction

Cervical cancer (CC) is the final stage of pre-defined precursor lesions, as the Low Grade-Squamous Intraepithelial Lesion (LSIL) followed by the High Grade-SIL (HSIL) and finally CC. It is the second place in incidence and mortality among female neoplasias worldwide [1]. These neoplasias could have several risk factors, among them, pregnancy number, alcohol and tobacco consumption, number of sexual partners, and,

the most related etiological factor, high risk-Human papillomavirus (hr-HPV) infection [2].

The transformation step is not a common occurrence of an HPV infection, and only a small number of cervical lesions infected by hr-HPV types evolve into CC. The immune response plays an important role in clearing most of these infections, but some infections cannot be eliminated and persist for several years, becoming an additional risk factor [3].

Two of the hallmarks of cancer are related to the immune system response, one of them related to the avoidance of the immune system recognition and another related to inflammation prompted by the tumor. These are mediated by a plethora of molecules such as cytokines like interferons (IFN) and chemokines among others [4].

Interferons are small single-chained glycoproteins, involved in the first line of defense against pathogens such as viruses, bacteria, and parasites. Interferons are classified in Type I (IFN α , - β , - ϵ , - κ , - τ and - ω) and Type II (IFN γ) upon receptor binding. Type I IFN binds to IFNAR1 and IFNAR2, while type II IFN binds to IFNGR1 and IFNGR2 [5].

Specifically, IFN ϵ gene is located in chromosome 9p21, gives rise to a single exon mRNA, and is translated to a 192 amino acid protein [5, 6]. IFN ϵ has found to be constitutively expressed in lung, brain, small intestine, and reproductive tissues. It exerts lower anti-viral, anti-proliferative, and natural cell killer enhancing activities compared to IFN α family members [5].

Immunological mechanisms play a key role in the etiology and progression of many and perhaps all cancers [7], elevated type I IFN is a component of the signature associated with chronic immune activation [8]. Although most women will be infected by HPV at some point, very few will progress to invasive disease [1]. The identification of more robust markers of the disease progression requires a more complete molecular and cellular characterization. At present reports about IFN ϵ expression in CC are scarce.

Therefore, we decided to evaluate the potential molecular alterations at DNA and RNA levels by *in silico* methods, and by means of RT-PCR at mRNA level and at protein level by immunohistochemistry and immunocytofluorescence in cervical tissue with the different lesions of the cervix.

Materials and methods

Chromosome 9 CNV in silico analysis

As previously reported (Marrero-Rodriguez 2017 data submitted) for copy number variation analysis, a total of 115 cancer libraries and 32 control libraries corresponding to

GSE10092 and GSE52904 were downloaded. Partek v6.6 (Partek Incorporated, Saint Louis, MO, USA) were used. Stringent parameters were set as follows: each segment must contain a minimum of 10 consecutive filtered probe sets, a *p*-value threshold of 0.001 when compared to the neighboring adjacent regions and a signal-to-noise threshold of 0.5. The cut-off value for the gain was set at above 2.3, while loss was set at below 1.7. CNV was called for the gains or losses that occurred in at least 10% of the total samples. The 22 somatic chromosomes and sexual chromosomes were analyzed. Hierarchical clustering was done by the following parameters, sample dissimilarity: Euclidian and cluster method: average linking.

Cervical cancer transcriptome in silico analysis

As previously reported (Marrero-Rodriguez 2017 submitted) a total of 8 normal (N) and 57 cervical cancer (CC) experiments were downloaded and analyzed. The data used for these analyses were downloaded from European Bioinformatics Institute (EMBL-EBL) and GEO. These data correspond to GSE7307, GSE5787, GSE3526 and 1 Gene Expression Atlas (GSE-2109) corresponding to Affymetrix Human Gene Chip U133 platform.

Data sets analyses were achieved by means of CEL files with the Expression Console, Partek Genomics Suite 6.6v software (Partek Incorporated, Saint Louis, MO, USA) and Transcriptome Analysis Console (Affymetrix, Santa Clara, CA, USA). Pearson and Spearman correlation was performed and probe sets were summarized by means of Median Polish and normalized by quantiles with no probe sets excluded from analysis. Background noise correction was achieved by means of Robust Multi-chip Average (RMA) and data were log₂-transformed. Data grouping and categorization was achieved by principal component analysis (PCA). Differentially expressed genes were detected by means of ANOVA. Genes were considered altered with +1.5 or -1.5 fold change, *P*≤0.05 and FDR>0.05 parameters.

Cervical tissue collection

Fifty nine cervical tissue samples (taken from patients aged between 18 and 74 years) including: three histological and colposcopic normal tissues, twenty LGSIL, sixteen HGSIL, and twenty carcinomas (16 squamous cell car-

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cinomas and 4 cervical adenocarcinomas), were collected from the Hospital General de Mexico in Mexico City, from May 2012 to May 2013. Detailed clinic-pathological information was obtained from the patient's records. Patients were recruited with signed informed consent and ethical approval from the local institutional board in accordance with the Helsinki declaration. Tissue samples were collected prior to the administration of any chemotherapy and/or radiotherapy treatments. These same cervical tissues were previously reported [9].

RNA extraction and reverse transcription reaction

Total RNA was extracted with the RNeasy tissue Mini Kit (Qiagen Inc, USA). The cervical tissue samples were disrupted and homogenized in 1 ml of Qiazol Lysis Reagent. Samples were then incubated at room temperature for 5 min. Next, 200 µl of chloroform was added, and samples were incubated at room temperature for 3 min. The mixture was centrifuged at 12,500 rpm for 15 min at 4°C. The aqueous phase was transferred to a new tube and mixed with an equal volume of 70% ethanol. Samples were then transferred to an RNeasy Column in a 2 ml tube, and centrifuged at 10,000 rpm for 15 sec. After centrifugation, 700 µl of RW1 buffer was added and mixture was centrifuged at 10,000 rpm for 15 sec. Flow-through was discarded and 500 µl of RPE buffer was added to the membrane and then centrifuged at 10,000 rpm for 15 sec (2×). The column was transferred to a new collection tube then 30 µl of RNase free water was added and centrifuged for 1 min at 10,000 rpm. RNA elution was measured in a Nanodrop-ND-1000 (Thermo Scientific, DE, USA) and RNA integrity was checked in 1.5% agarose gel. After purification, 1 µg of total RNA was retro transcribed in a 20 µl final volume reaction with the SuperScript VILO Master Mix (Applied Biosystems, CA, USA) 4 µl of Master Mix were added, and the reaction mixture was incubated at 25°C for 10 min., 42°C for 60 min., and 85°C for 5 min., according to manufacturer protocols.

IFN epsilon transcripts detection

Specific IFNε primers were in-house designed using primer BLAST software found on the NCBI website, with the NCBI reported sequences and RPS18 were used as internal control, previ-

ously reported. Sequences were as followed: IFNεF 5'-CCA AAA AGC ACA CAC TCT GGC-3', IFNεR 5'-CCG TGT GGT TTT CCT CCC AA-3', RPS18F 5'-AATCCACGCCAGTACAAGATCCCA-3' and RPS18R 5'-TTTCTTCTTGACACACCCACGGT-3'. For end point PCR, the conditions were as follows: 12.5 µl of GoTaq® Green Master Mix (Promega, WI, USA), 1 µl of cDNA template, and 20 pmol of each primer, in a 25 µl total volume reaction, with the following program: 45 seconds at 95°C, 30 seconds at 60°C and 30 seconds at 72°C for 30 cycles. Endpoint PCR products were resolved in 2% ethidium bromide stained agarose gel. The intensity of each band was analyzed employing Kodak 200 Molecular imaging analysis software and densitometry assay was carried out.

DNA extraction and HPV detection and genotyping

DNA from cervical tissue samples was extracted from the phenol phase of the RNA extraction procedure, incubated for 5 min with 300 µl 100% ethanol at room temperature, centrifuged at 10,000 rpm for 5 min. Resulting pellet was washed with 1 ml sodium citrate (0.1 M sodium citrate in 10% ethanol, pH 8.5) for 30 min at room temperature and then centrifuged at 10,000 rpm for 5 min (3×). DNA was washed with 75% ethanol and centrifuged at 10,000 rpm for 5 min, pellet was air-dried, and DNA resuspended in nuclease free water. HPV detection was accomplished using oligos GP5+/GP6+ as described [10], conditions were as follows: 12.5 µl GoTaq Green Master Mix, 20 pmol of each primer, and 100 ng DNA in a 25 µl final volume. PCR program used was 40 cycles of 94°C for 30 sec, 55°C for 1.5 min, followed by 72°C for 1.5 min. PCR products were resolved in 2% ethidium bromide stained agarose gel. The intensity of each band was analyzed employing Kodak 200 molecular imaging analysis software. HPV genotyping was carried out using Linear Array® Genotyping Test (Roche, IN, USA) and HPV 2 CLART® (Genomica, Madrid, Spain) according to manufacturer's instruction.

IFNε immunohistochemistry assay

A Tissue microarray (TMA) of the 59 cases was constructed as follow. Sections from paraffin-embedded, formalin-fixed tissue blocks were stained with hematoxylin-eosin and reviewed

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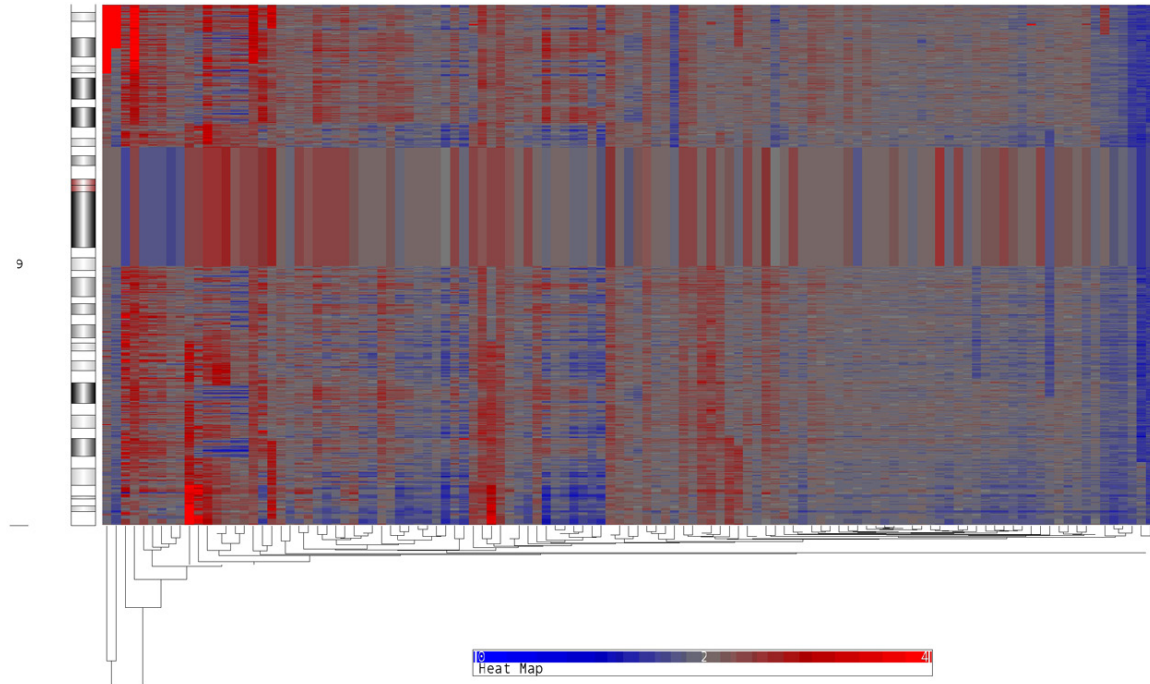


Figure 1. Heatmap of the chromosome 9 in cervical cancer. Hierarchical clustering of one hundred and fifteen cervical cancer copy number variation results of the chromosome 9. The 9p21 cytogenetic region where IFN ϵ is coded shows no significant alterations.

by a pathologist to select areas of invasive tumor. Core samples were taken using 0.6 mm² blunt-tip needles and were placed on the recipient microarray block using a Tissue Microarrayer (Chemicon Co., MA, USA). Tumors were represented with 2-fold redundancy, which has been shown to provide a sufficiently representative sample. Sections (3 μ m) were cut and placed onto coated slides. TMA slides were deparaffinized with xylene followed by ethanol and rehydrated. Immunostaining was performed using a streptavidin-biotin complex peroxidase method (Dako, Glostrup, Denmark).

Briefly, after de-waxing the sections, endogenous peroxidase activity was inhibited with freshly prepared 0.5% H₂O₂ in distilled water for 20 min. Next, the sections were processed in a 600 W microwave oven at maximum power, three times for 5 min each in Tris-EDTA buffer (pH 9.0). Incubation with polyclonal rabbit anti-IFN ϵ (NBP1-92018; Novus Biologicals, CO, USA), was performed overnight at 4°C in a humidity chamber, at 1:100 dilution, in 1% Bovine serum albumin (BSA). Sections were developed with a peroxidase substrate solution (0.05% 3,3-diaminobenzidine tetrahydrochloride,

0.01% H₂O₂ in PBS), counterstained with haematoxylin, dehydrated, and mounted. Testis and placenta tissues were used as positive biological controls, and negative controls consisted of the replacement of the primary antibody with 1% BSA. Three independent observers performed assessment of IFN ϵ expression at independent times by light microscopy at 20 \times magnification. Immunostaining was evaluated as negative and positive staining.

IFN ϵ Immunocytofluorescence in CC cell lines

SiHa, HeLa and C33A cells were grown on 5 mm coverslips (previously treated with poly-D-Lys). Cells at 70% of confluence were fixed with 4% paraformaldehyde in PBS 1 \times during 30 min. Fixed cells were washed (3 times) with PBS 1 \times . Cells were permeabilized with PBS1 \times -Triton 0.2% (200 μ l of Triton X-100 in 100 ml of PBS 1 \times pH 7.4) during 30 min. To block unspecific binding of the primary antibody BSA in PBS 1 \times was used (10 mg/ml BSA in PBS 1 \times pH 7.4-Triton 0.2%) at room temperature during 1 hr. Overnight incubation at 4°C of the primary antibody anti-IFN ϵ (NBP1-92018; Novus Biologicals, CO, USA), 1:200 dilution was used. After

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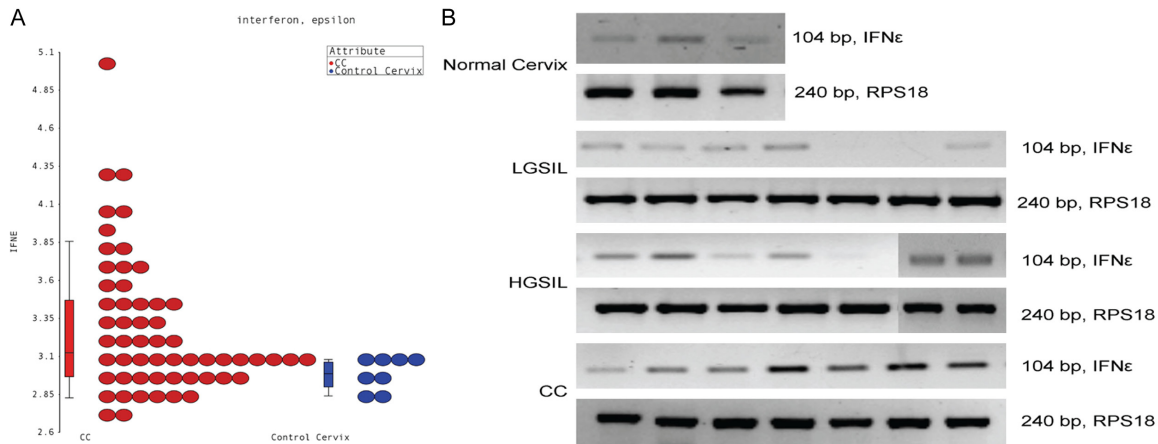


Figure 2. Interferon epsilon mRNA expression in cervical tissue. A. Shows dot blot from the IFNε gene expression pattern in cervical cancer and normal cervical tissues, with red dots representing CC and blue dots representing the normal cervix. B. Consists in four panels corresponding to normal, LGSIL, HGSIL and CC; each panel present two agarose gel electrophoresis, upper gel corresponds to IFNε (104 bp) PCR product and lower gel corresponds to internal control, the constitutive expressed RPS18 (240 bp). IFNε expression in normal cervical tissue was considered as basal with presence of faint band. LGSIL present the same band intensity as normal cervix in 16/20 tissues analyzed, representation of five of these are presented, and two of the 4/20 negative to amplification. The HGSIL panel shows the five with marked band intensity, while the remaining presented a similar band intensity or negative amplification. Increased band intensity was observed in 16/20 CC samples, representation is showed in seven samples analyzed and one showing same band intensity as the normal cervix.

IFNε antibody incubation, three PBS 1x-Triton 0.2% washes was done. Incubation of 1 hr with the secondary antibody goat anti-rabbit Alexa488 (A11034, Abcam, MA, USA) at 1:500 dilution was done protected from light, followed by three PBS 1x-Triton 0.2% washes. Finally, the nucleus was stained with 4'6-diamidino-2-phenylindole (DAPI) at 10 μg/ml during 10 min and three PBS 1x-Triton 0.2% washes. Finally coverslips were mounted using Fluorogel (17985-10, Electron Microscopy Sciences).

Statistical analysis

Clinical and pathological relation analysis was performed by means of the ANOVA and X² tests for IFNε expression. All *p* values represent two-tailed tests and were considered significant at 0.05. Statistical analysis was performed using SPSS v15 statistical software.

Results

Interferon epsilon copy number variation and expression *in silico* molecular analyses

Our first approach was to perform copy number variation and expression analysis using the available cervical cancer microarray libraries.

The copy number variation results showed that ~10% (11/115) gained the 9p21.3, IFNε gene coding region, whereas ~3% (4/115) lost it and the remaining 87% (100/115) showed no alteration (**Figure 1**). Meaning that there were no significant CNV alterations at DNA level of the IFNε gene.

On the other hand, *in silico* expression analysis showed that there is an up-regulation of the IFNε mRNA (**Figure 2**). Following these results we decided to validate the *in silico* findings by end point-PCR.

Interestingly these results potentially tells us that up-regulation of the IFNε gene could not be related to copy number, but instead by epigenetic mechanisms, which must be validated by proper experiments.

IFNε mRNA expression in cervical tissue

First, we observed a positive faint band in the normal tissues, and this was considered as basal expression (value 1) and then compared against LGSIL, HGSIL and CC samples. In the LGSIL group, 16/20 present amplification with similar densitometric values as normal tissue, near one, while the remaining 4/20 did not show any amplification. Most of the

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Table 1. Statistical correlation between IFN ϵ expression and clinical features

Clinical Variables	Average \pm SD	IFN ϵ (<i>p</i> value)
Age	40.44 \pm 15.11	0.07
\geq 50	14	
<50	45	
Pregnancies	3.31 \pm 2.6	0.05*
\geq 3	29	
<3	30	
HPV single or multiple infection		0.90
1	21	
2	10	
3 or more	5	
Negative or not identified	13	
Menopause	45.78 \pm 4.94	0.06
\geq 45	13	
<45	46	
Tobacco consumption		0.03*
Positive	16	
Negative	43	
Alcohol consumption		0.86
Positive	13	
Negative	46	
Contraceptives		0.03*
Positive	30	
Negative	26	
Diagnosis		0.0001*
CC	20	
Non CC	36	

*Statistical significance.

HGSIL biological samples analyzed resulted positive on IFN ϵ expression. Meaning that, only 2/16 did not show any amplification, and similar band intensity to LGSIL was observed in 9/16, whilst 5/16 from this group showed marked band intensity, densitometric assays (data not shown) corroborate this observation. Interestingly, in CC 2/20 did not show any amplification, 2/20 showed similar band intensity to normal tissue, and the remaining 16/20 showed a marked augmented presence of the IFN ϵ mRNA band (**Figure 2**). Our results suggest that IFN ϵ up-regulation in cancer ($P=0.0001$) is an event associated primarily to cellular transformation rather than an HPV-induced event. Apparently up-regulation is present as an independent event from single or multiple HPV infection ($P=0.90$) and as for the presence of HPV specific genotype infection ($P=0.29$) (**Table 1**).

IFN ϵ protein in cervical tissue by IHC

In order to corroborate if the increased mRNA level was due to an up-regulation in gene expression or if it was related to the number of cells expressing the gene we carried out immunohistochemistry of the IFN ϵ protein. We observed that IFN ϵ protein immunostaining was primarily present in the epithelial basal cells. Also, less intense signal was present in supra-basal and granular strata and expression was primarily in the proliferative non-differentiated strata cervical cells in the normal cervical tissue. The same IFN ϵ protein expression pattern was observed in the LGSIL, in which the immunostaining was primarily in the basal, suprabasal and less differentiated layers of the cervical epithelium. Interestingly, samples from the CC group, displayed a positive immunostaining in all transformed epithelial cells (**Figure 3**). IFN ϵ expression was not limited to the epithelial cells, but also in the vascular endothelium. In other words, most of the tissues corresponding to CC showed positive expression. Interestingly, the tissues analyzed for IFN ϵ protein expression showed a similar intensity in expression at the protein level. This could possibly mean that the augmented presence of the mRNA might not necessarily represent an overexpression of the gene, but rather, an increased number of cells expressing the gene.

IFN ϵ in cervical cancer cell lines

To corroborate the IFN ϵ expression regardless of the HPV genotype and/or infection observed in cervical tissue, immunocytofluorescence in HPV16 (SiHa), HPV18 (HeLa) and HPV- (C33A) cell lines was carried out. We observed positive immunostaining of IFN ϵ protein primarily in cytoplasm in the three cell lines in disregard of the HPV status (**Figure 4**).

Discussion

IFN ϵ is a relatively new described gene [6]. In the present work, we analyzed the mRNA and protein expression of this molecule in different

IFN ϵ cytokine expression in cervical cancer

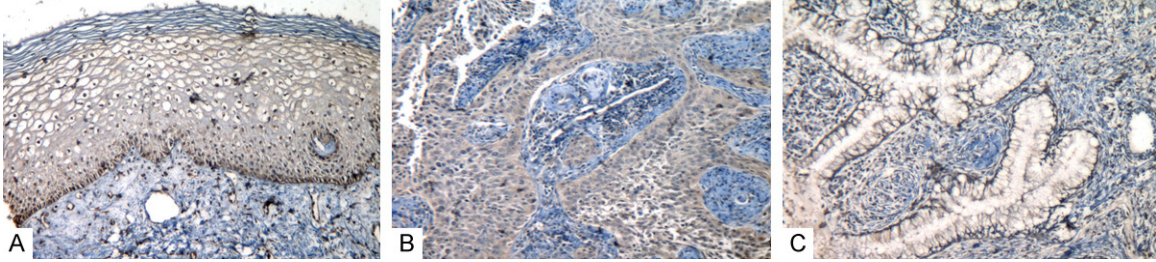


Figure 3. Interferon epsilon protein was present in CC. Normal cervix (A) showed positive strong cytoplasmic immunosignal in the basal strata, also positive cytoplasmic signal in the parabasal and proliferating stratum, negative immunosignal in the more differentiated, external strata. In contrast, in CC, in both histological types, meaning that, adenocarcinomas (C) and squamous (B) cervical cancer was positive for cytoplasmic immunosignal for IFN ϵ , potentially correlating with its presence with proliferative cells.

cervical tissues, ranging from healthy cervical tissue throughout the LGSIL, HGSIL, and finally the invasive stage of the neoplasia. Interesting results were obtained. Disregard of the gene copy number and HPV infection or absence, we observed increased presence at mRNA as lesion degrees were higher and protein presence with cytoplasmic and nuclear localization were found. We report no significant IFN ϵ gene copy number alterations in CC, suggesting that epigenetic or additional mechanisms could be implicated in its transcriptional regulation.

Interferons are a family of α -helical cytokines that, once secreted, bind to glycosylated, species-specific, cell trans-membrane proteins that trigger signaling through their cytoplasmic domains. The activated cytoplasmic domain, in turn activates Janus Activated Kinase (JAK), Signal Transducers and Activators of Transcription (STAT), Mitogen Activated Protein Kinase (MAPK), Phosphatidylinositol-4,5-biphosphate 3 Kinase (PI3K). This kinase-activation leads to IFN-Stimulated Gene Factor 3 (ISGF3) binding to IFN-Stimulated Response Elements (ISRE's). Interferons through their kinase-driven signaling, lead to the induction of over 2000 transcriptionally regulated IFN stimulated genes (ISG) with varying patterns of temporal expression. Through their cellular actions, IFN can alter the emergence, progression and regression of malignancies [11, 12].

Type I IFN's exhibit a broad spectrum of biological activities and can influence viral response, proliferation, and modulate cytotoxic activity in cells of the immune response system such as T cells, NK cells, monocytes, macrophages and dendritic cells. They can modify expression of

anti-apoptotic genes such as B-Cell Lymphoma 2 (BCL2), and pro-apoptotic genes like BCL2 Agonist Killer (BAK) and BCL2 Associated X protein (BAX), it can modulate differentiation and angiogenic activity [13].

The IFN γ family member expression is shown to be down-regulated in CC tissue in comparison with healthy tissue [14]. E6 HPV protein is able to reduce IFN α and IFN β mRNA levels [15]. Our results show that IFN ϵ presents a behavior dissimilar to the related IFN type I family members, since it presents mRNA augmented signal throughout the lesions. In other words, a gradual increase in expression of the IFN ϵ mRNA occurs as the natural progression of the disease advances. This could indicate that the rise in the expression of this molecule could be an important event during early carcinogenesis. Making it a promising molecular marker of neoplastic progression.

At protein level, a similar expression pattern was observed for this gene in healthy epithelium from the vagina and cervix, in which expression was observed in the proliferative cells layers of the basal, para-basal strata [16, 17]. The expression pattern could represent a marker for less differentiated cells or proliferative stages. In CC, it could represent a dedifferentiation marker, or as well a proliferation marker, since it has been previously described with higher levels of expression in proliferative stages [18]. This is due to the fact that basal cell strata of the cervical epithelium are primitive cells committed to proliferation instead of differentiation in contrary to the upper layer that are more differentiated committed cells, comparing them to the proliferative, poorly differentiated transformed cells in cancer. Although, we observed

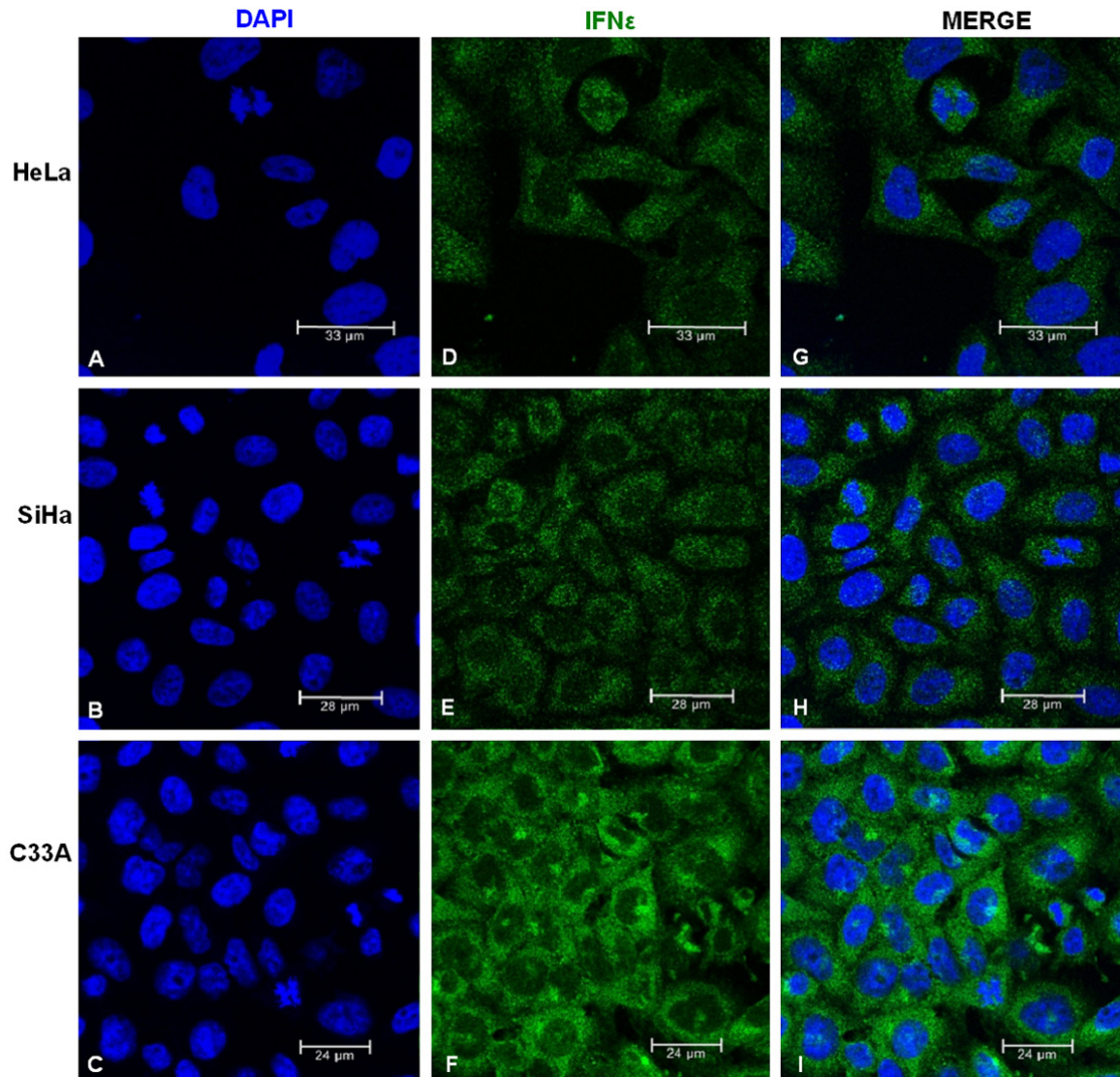


Figure 4. Protein expression in distinct HPV-status cervical cancer cell lines. Three cervical cancer cell lines were used. First, SiHa correspond to a HPV16 squamous cell carcinoma, HeLa correspond to an HPV18 cervical adenocarcinoma and C33A which correspond to a HPV- squamous cell carcinoma. (A-C) Show HeLa, SiHa and C33A (respectively) DAPI nuclear staining. (D-F) Show the positive, cytoplasmic IFN ϵ immunosignal in the three cell lines. (G-I) Show the merged image from panels (A-F). Positive immunosignal was observed in the three cell lines regardless HPV status.

positive immune signal in endothelial and fibroblast cells.

Although the IFN ϵ pathway and biological function are not fully understood, further research is needed. A report showed that CC cell line HeLa express constitutively IFN ϵ and no other IFN family member. In addition, IFN ϵ shows diminished expression leading to attenuated expression and phosphorylation of STAT1 and down regulation of the Retinoic Acid-Inducible Gene 1 (RIG1) expression [19].

Although IFN may be important for cellular homeostasis and resistance to cancer growth, production of endogenous IFN can also contribute to pathology. Increased expression of ISG has been identified in cancer cells compared to corresponding normal primary cells or normal tissues with correlation to the degree of tumor invasion [11]. This could potentially correlate with our results, where low IFN ϵ expression levels in healthy tissue may be produced continuously and remain localized with no systemic effect [11], while in CC up-regulation of IFN ϵ

gene expression could play an important role. Up-regulation of IFNε expression has been associated to proliferation, and regulated by sex hormones, particularly by estrogen receptor [18], which is expressed in CC [20].

In conclusion, an increase in IFNε mRNA could represent a promising molecular marker in CC cells that could have an important role in cellular homeostasis and could influence immune related events in cervical carcinogenesis. Additionally, IFNε expression was not related to HPV infection or genotype, and potentially CNV does not affect IFNε gene expression in CC, suggesting that it could be regulated by epigenetic mechanisms. Further research is needed to fully elucidate the role of IFNε role in CC. We are currently approaching this issue by more thorough experiments, and the possible validation of molecular marker as diagnostic or prognostic is also being addressed by increasing sample number as well as improved patient follow up.

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Disclosure of conflict of interest

None.

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