

Galectin-9 Expression is Correlated to Cervical Squamous Cell Carcinoma Progression and Overall Survival

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Purpose: To determine whether galectin-9 gene (*LGALS9*) expression is correlated with cervical cancer progression, clinicopathological characteristics, and overall survival. To determine the biological processes and the abundance of tumour infiltrating immune cells related to the expression of *LGALS9*.

Patients and Methods: The study was conducted in two phases: 1) The expression level of *LGALS9* was determined using the data of 193 squamous cell carcinoma (SCC) samples from The Cancer Genome Atlas (TCGA) database. Biological processes and tumour infiltrating cells associated to *LGALS9* expression were evaluated using gene set enrichment analysis (GSEA) and tumour immune estimation resource (TIMER). 2) Independently, galectin-9 was identified in 40 SCC samples by immunohistochemistry and optical density quantified using ImagePro[®] software.

Results: The *LGALS9* gene showed increased expression in cervical cancer samples. A higher expression level in SCC was related to better overall survival and to early clinical stages. GSEA showed that tumours with higher expression of *LGALS9* were enriched in immune pathways such as interferon_alpha_response, and complement, the analysis of TIMER database showed a positive correlation between the expression level of *LGALS9* and the abundance of tumour infiltrating immune cells. In addition, higher expression of galectin-9 was found in biopsies of SCC patients at early clinical stages, showing a trend of better survival.

Conclusion: Higher expression levels of *LGALS9* and galectin-9 in SCC were related to early clinical stages and better prognosis. GSEA and TIMER analysis suggested that galectin-9 could play an antitumor role in cervical SCC.

Keywords: biomarker, galectin, cervical neoplasia, immune response, clinical stage, overall survival

Introduction

Cervical cancer is the fourth most common cancer in women worldwide, and the incidence and mortality rates differ drastically between low-income and high-income countries. Most cases occur in low-income countries, where many women are diagnosed in advanced stages, leading to higher mortality rates.¹

Lack of opportune diagnosis and effective medical treatment are the main causes of high mortality due to cervical cancer. Therefore, it is still necessary to find new therapeutic options that improve the survival of women with cervical cancer.² Immune cells play important roles in the vigilance and elimination of malignant cells, but evading tumour cells secrete an array of soluble factors including cytokines, chemokines and growth factors that modify surrounding normal and immune cells to promote an immunosuppressive state that favour cancer development. Tumour cells act on immune cells by inducing several regulatory pathways including, but not limited to, checkpoint molecules, a group of proteins

that control the timelapse and intensity of immune response, such as cytotoxic T-lymphocyte protein 4 (CTL4), programmed cell death protein 1 (PD-1) and T cell immunoglobulin and mucin domain-3 (TIM-3). Current cancer therapies are directed against checkpoint proteins to restore the protective role of immune cells.^{3,4}

Galectins constitute a family of glycan-binding proteins that play central roles in the reprogramming of immune cells in the tumour microenvironment (TME). There have been recognized at least 15 Galectins (Gal-1 to Gal-15) that share the capacity to interact with β -galactoside-containing glycoconjugates but differ in their fine specificity and their biological activities. Galectins may behave as pro-tumoral (Gal-1) or antitumoral factors (Gal-3) and can be up- or down-regulated during cancer development.^{5,6}

One of the most intriguing members of the group is Galectin-9, it is expressed in different epithelial tumours, including cervical carcinoma.^{7–10} Galectin-9 is a tandem repeat protein with two nonhomologous carbohydrate recognition domains that bind to different oligosaccharide structures.¹¹ Galectin-9 can play several roles depending on its interaction with different ligands and can promote or inhibit tumour activity.¹² It has been shown that galectin-9 participates in tumour cell adhesion, aggregation, migration, apoptosis, and growth.^{9,13,14}

TIM-3 is the main receptor of Galectin-9, it is expressed on the membrane of both immune and tumour cells. The interaction of TIM-3 with galectin-9 induces apoptosis of Th-1 cells and is involved in the evasion capability of cancer cells.^{15–17}

Galectin-9 may exert antitumour effects in different cancer types. In breast cancer, galectin-9 induces tumour cell aggregation and prevents metastasis, these effects correlated with increased survival.^{18,19} In colorectal cancer, galectin-9 induces apoptosis and suppresses proliferation;²⁰ consequently, decreased expression of galectin-9 has been associated with poor outcome.²¹

Previous studies showed high galectin-9 expression in cervical cancer patients without lymph node metastasis, showing a trend towards improved survival.^{10,22} Contradictorily, cervical cancer patients positive for human papilloma-virus showed higher levels of TIM-3 and galectin-9 that promote Treg cell differentiation that inhibits the cytotoxic activity of CD8+ T cells.²³

The roles of galectin-9 in tumour cells suggest that galectin-9 could be a potential therapeutic target for the treatment of cervical cancer. Therefore, studying the expression of *LGALS9* and the coding protein galectin-9 in the progression of cervical cancer will provide valuable information on its potential use as a therapeutic molecule.

The purpose of this work was to evaluate the association of galectin-9 expression with clinicopathological characteristics, and overall survival in cervical cancer patients. To identify the biological processes and the abundance of tumour infiltrating immune cells related to the expression of *LGALS9*.

Materials and Methods

Database Analysis

Data were obtained from the TCGA TARGET GTEx study included in the XENA browser (<https://xenabrowser.net/>). As a preliminary analysis, the RNAseq database was utilized to compare the mRNA expression levels of the galectins *LGALS1*, *LGALS2*, *LGALS3*, *LGALS4*, *LGALS7*, *LGALS8*, *LGALS9*, *LGALS12*, *LGALS13* and *LGALS14* between normal cervical tissue (n = 10) and squamous cell carcinoma (SCC), (n = 193) or adenocarcinoma (AC), (n = 37). The expression levels of mRNA were evaluated to determine if they were associated with overall survival. Because only *LGALS9* expression was associated with overall survival in SCC, the study focused on *LGALS9* expression in SCC and its relationship with clinicopathological characteristics. For this, a selection of samples from TCGA Cervical Cancer (CESC) was analysed considering the following criteria: primary site, sample type, and histological type, this analysis only included SCC samples (n = 183) (Table 1).

To determine the biological processes related to the expression of *LGALS9*, a gene set enrichment analysis (GSEA) was performed, with samples grouped into high or low mRNA levels according to median expression. The Molecular Signatures Database v2023.1 was used to select hallmark gene sets. The number of permutations performed was 1000. A NOM p value < 0.05 and an adjusted q-value FDR < 0.25 were considered significant.

Table 1 Characteristics of Patients with Cervical SCC Included in the RNAseq Analysis and in the Immunohistochemistry Assay

Clinico-Pathological Characteristics	SCC Samples of the TCGA Database	SCC Samples Included in Immunohistochemistry Assay
Age range	24–88 (years)	26–79 (years)
Mean age	49 (years)	53 (years)
Histological type	Number of cases	Number of cases
Squamous cell carcinoma	193*	40
Clinical stages		
I	69	8
II	71	11
III	33	13
IV	14	8
Pathological grading		
G1	7	0
G2	85	22
G3	73	8
G4	1	5
Keratinizing		
NKSCC*	80	8
KSCC**	40	27

Notes: The number of samples used for the different analysis varied depending on the information available in the database. *Non Keratinizing Squamous Cell Carcinoma; **Keratinizing Squamous Cell Carcinoma.

Additionally, to evaluate the correlation between the mRNA of the *LGALS9* gene and tumour infiltrating immune cells, we analysed the data included data of the Tumor Immune Estimation Resource (TIMER) database (<https://cistrome.shinyapps.io/timer/>).

Patients and Samples

The study was conducted in accordance with the Declaration of Helsinki and the ethical regulations approved by the Human Ethics Committee 785 from the Mexican Institute of Social Security (IMSS), registration number (R-2017-785-119).

The female patients included in the study were invited to participate at the Radiotherapy Service of the Unidad Médica de Alta Especialidad-Hospital de Especialidades (UMAE-HE), IMSS, at Puebla City, Mexico, during the period February 2017 to February 2022. All patients included in the study signed the informed consent form. The clinicopathological characteristics were obtained from clinical records. Patients included in the study did not receive prior treatment.

Galectin-9 Detection in Cervical Cancer Tissue

Paraffin-embedded biopsies of SCC of the cervix were obtained from the Department of Pathology of the UMAE-HE, IMSS. A total of 40 biopsies were included in the study, and the clinicopathological characteristics were obtained (Table 1). Histological Sections 4 µm thick were cut and placed on slides treated with (3-aminopropyl) triethoxysilane (APES) (440,140 Sigma Aldrich, Saint Louis, MO, USA); next, they were deparaffinized and rehydrated. Afterwards,

antigen retrieval was conducted by incubation with Tris-Buffer Saline for 15 min at 90°C. Every step was followed by washing three times with 1x PBS, except after incubation with blocking buffer. Sections were incubated with 0.3% H₂O₂ in 1x PBS for 15 min to block endogenous peroxidase activity. Next, the tissues were incubated with blocking buffer containing 5% bovine serum albumin (P6154, Biowest, Riverside, MO, US) in 1x PBS for 1 hour. Next, the sections were incubated at 4°C overnight with the anti-Galectin-9 primary antibody (54,330, Cell Signalling, Massachusetts, USA) at a dilution of 1:250 in PBS containing 1% bovine serum albumin.

Afterwards, the tissues were incubated for 1 hour with anti-rabbit IgG-HRP (ab6721, Abcam, Cambridge, UK) at a dilution of 1:1000. The colorimetric reaction was assessed with ImmPACT[®] DAB Peroxidase Substrate (SK-4105, Vector Labs, Burlingame, CA, US). Nuclei were counterstained with haematoxylin (HX87960674 Merck, Darmstadt, Hesse, DE), and the slides were mounted with VectaMount[®] Permanent Mounting Medium (H-5000, Vector Labs, Burlingame, CA, UU).

Immunostaining was scored with Image-Pro software (Media Cybernetics, Inc., Rockville, MD, US). For each sample, 5 photos were taken, and the staining density was determined in 5 areas of each photo. The results corresponded to the mean optical density of all values and was reported as galectin-9 protein expression levels. The galectin-9 expression level was evaluated to determine its relationship with the clinicopathological characteristics of the patients and overall survival.

Statistical Analysis

To compare the mRNA levels of *LGALS* genes between normal cervical tissue and cervical cancer tissue, a one-way ANOVA test of a factor or Kruskal–Wallis test was used according to the normality of the data, along with a Bonferroni or Dunn's multiple comparisons post-hoc test, respectively. To determine whether the mRNA and galectin-9 expression levels were related to the survival of the patients, Kaplan–Meier curves and a log rank analysis were used.

Univariate and multivariate Cox regression analyses were performed to determine the association between *LGALS9* expression and several variables, including clinical stage, pathological grading, keratinization, and overall survival.

To identify the enriched groups of genes in the samples with high levels of the *LGALS9* gene, GSEA was performed with GSEA_4.3.2 software. To determine whether the mRNA and protein expression levels of *LGALS9* were related to the clinicopathological features, Student's *t* test or the Mann–Whitney *U*-test was used depending on the normality of the data. A significance level of $\alpha < 0.05$ was used, and the statistical tests were performed in GraphPad Prism 10.0.0.

A Spearman correlation was performed for the abundance of tumour infiltrating immune cells and expression level of *LGALS9* gene.

Results

RNA Levels of Galectin Genes in Cervical Cancer vs Normal Tissues

The changes in the mRNA levels of galectin genes (*LGALS1*, *LGALS2*, *LGALS3*, *LGALS4*, *LGALS7*, *LGALS8*, *LGALS9*, *LGALS12*, *LGALS13* and *LGALS14*) in SCC and AC samples compared with normal tissue were determined using the RNAseq database from TCGA TARGET GTEx. *LGALS1* expression showed a slight reduction in SCC and more evident in AC compared with normal tissues (Figure 1A). *LGALS2* increased notably in both SCC and AC compared to normal samples (Figure 1B). *LGALS4* expression decreased in SCC but increased in AC samples (Figure 1D). *LGALS7* expression increased in SCC but did not differ in AC compared to normal tissues (Figure 1E). *LGALS9* expression increased in SCC and AC ($p = 0.0002$ and $p < 0.0001$ respectively). Taken together, higher expression of *LGALS3*, *LGALS4* and *LGALS9* was found in AC than in SCC samples (Figure 1C, D and F), while *LGALS1* and *LGALS7* expression was higher in SCC than in AC. *LGALS8* and *LGALS12* expression did not show significant changes in cervical cancer (data not shown). *LGALS13* was not detected in normal samples while *LGALS14* was detected in a limited number of samples (data not shown); therefore, they were not further analysed.

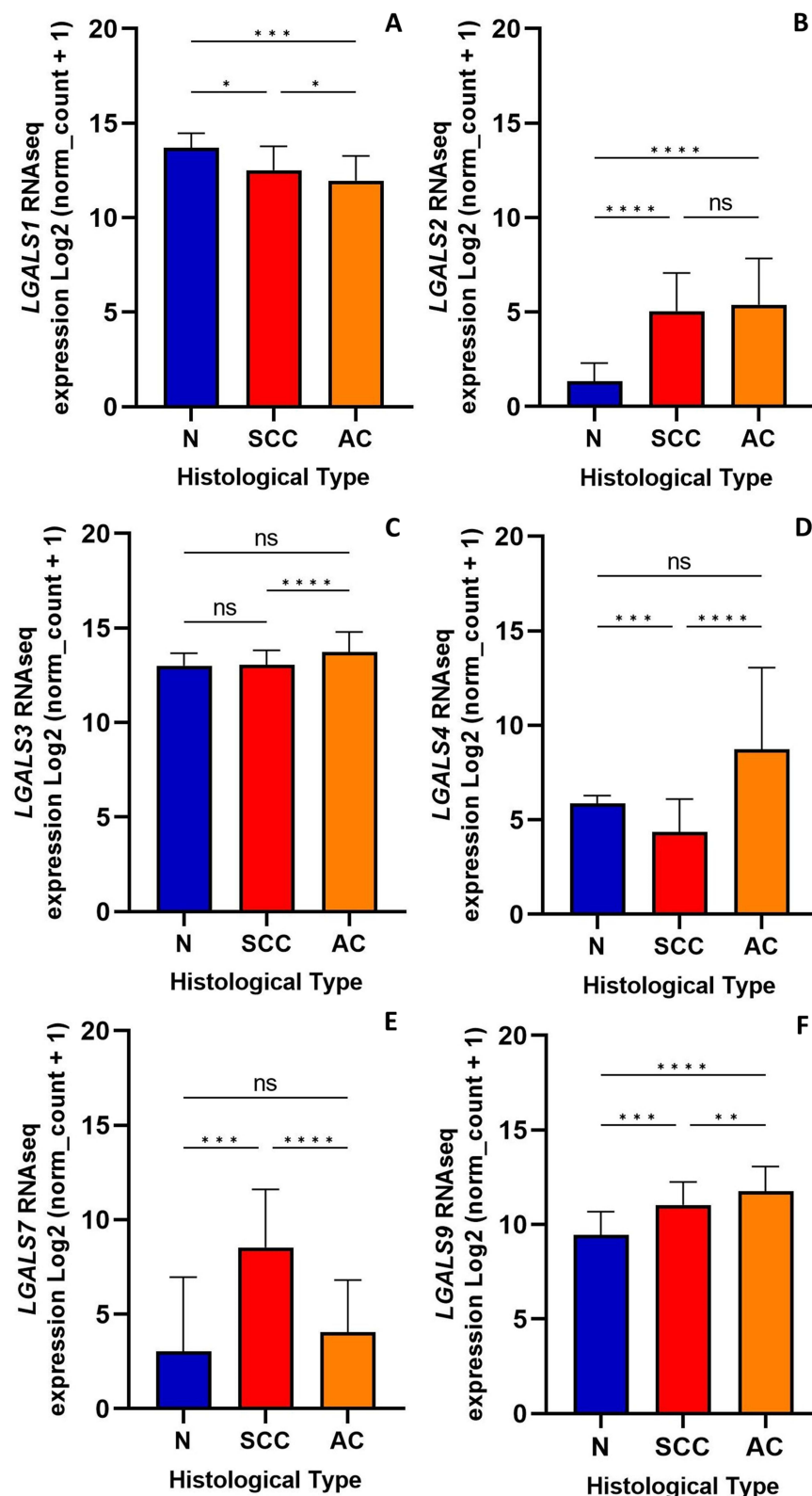


Figure 1 Expression levels of LGALS genes in cervical normal tissue (n=10) vs squamous cell carcinoma (n=193) and cervical adenocarcinoma (n=37). **(A)** Decreased expression of *LGALS1* in squamous cell carcinoma and adenocarcinoma. **(B)** Increased expression of *LGALS2* in squamous cell carcinoma and adenocarcinoma. **(C)** *LGALS3* did not show expression changes between normal and cervical cancer tissues. **(D)** *LGALS4* exhibited decreased expression in squamous cell carcinoma. **(E)** *LGALS7* exhibited increased expression in squamous cell carcinoma. **(F)** *LGALS9* exhibited increased expression in both squamous cell carcinoma and adenocarcinoma. For the genes *LGALS1* and *LGALS9*, the statistical analysis performed was one-way ANOVA and Bonferroni's multiple comparisons test, and for *LGALS2*, *LGALS3*, *LGALS4*, and *LGALS7*, the statistical analysis performed was Kruskal–Wallis and Dunn's multiple comparisons test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

Abbreviation: ns, non-significant.

RNA Levels of LGALS9 and Its Association with Overall Survival and Clinicopathological Characteristics

Further bioinformatic analysis were performed to identify the changes in *LGALS* genes expression associated to the overall survival. For this, *LGALS* genes that showed significant differences in mRNA levels in cervical cancer compared with normal tissues were divided into high and low expression groups, considering the median. Only for the *LGALS9* gene it was observed a statistical significance, higher levels of the *LGALS9* gene ($p < 0.0001$) were associated with better survival in patients with SCC (Figure 2A). There was no association between *LGALS9* expression and survival in patients with AC ($p = 0.9726$; Figure 2B).

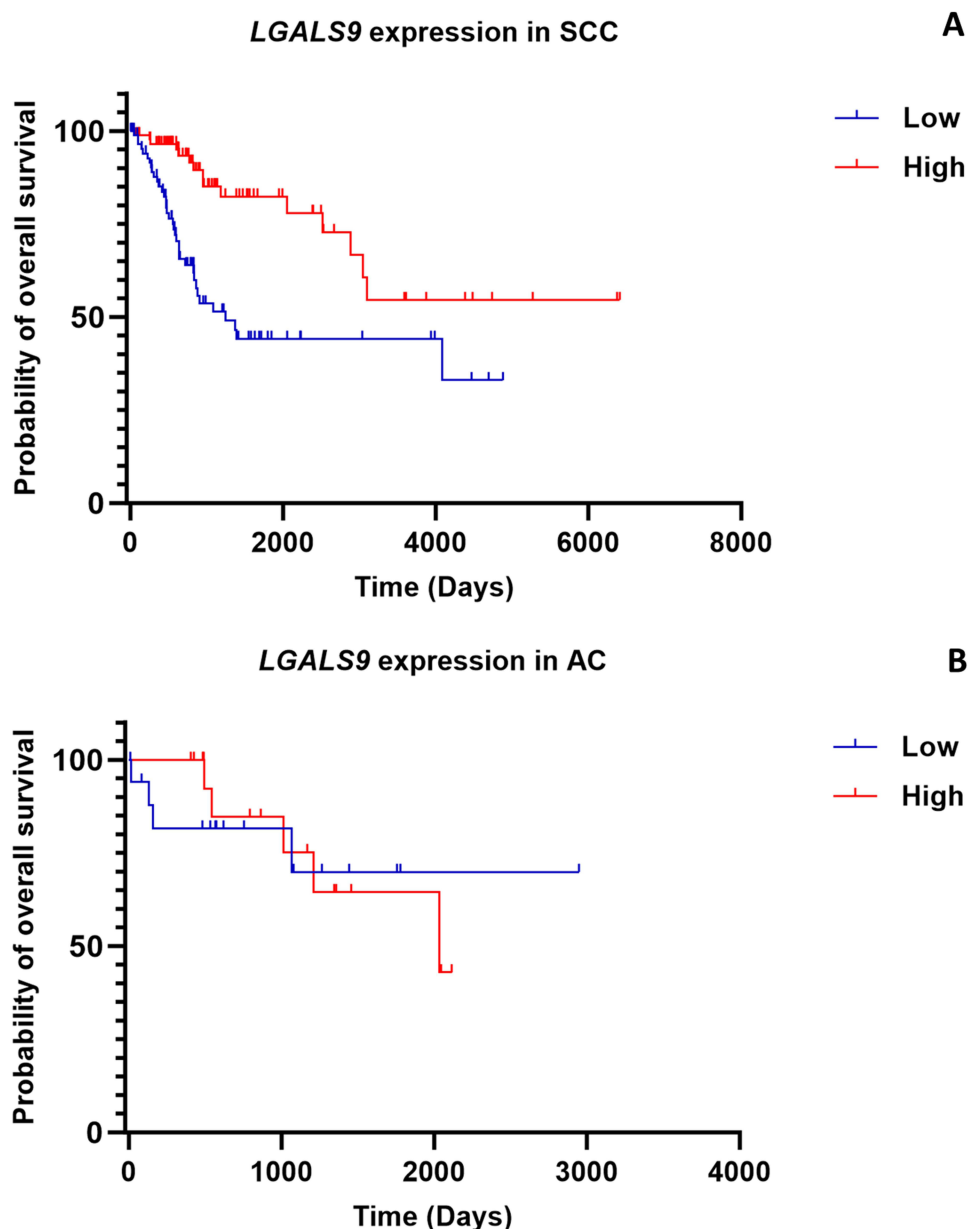


Figure 2 Expression level of *LGALS9* mRNA and its relationship with overall survival in cervical cancer patients. **(A)** Overall survival of patients with a diagnosis of squamous cell carcinoma with high ($n=91$) and low expression of *LGALS9* ($n=92$). **(B)** Overall survival of patients with a diagnosis of cervical adenocarcinoma with high ($n=17$) and low ($n=18$) expression of *LGALS9*. The groups of high and low expression for squamous cell carcinoma and adenocarcinoma were formed considering the median values (11.10 and 12.0, respectively). Kaplan-Meier and Log rank test analyses were performed to determine overall survival, considering $p < 0.05$.

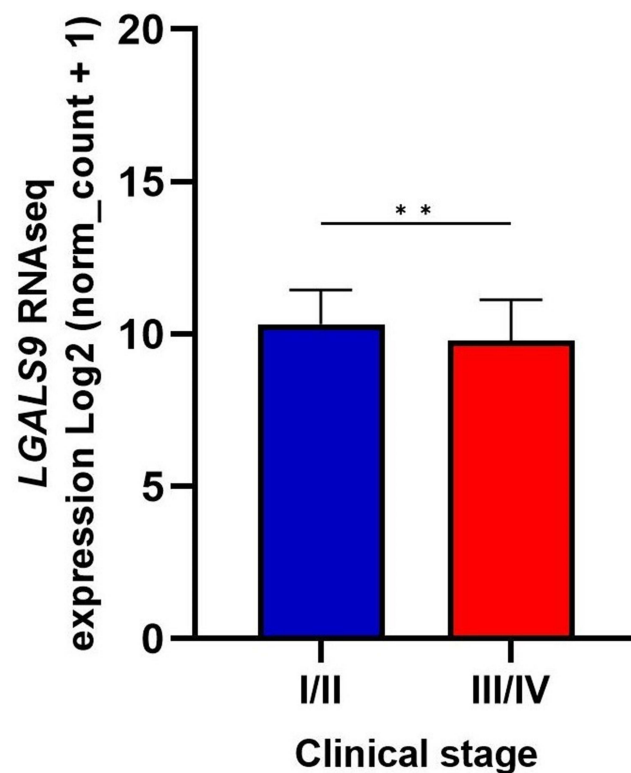


Figure 3 Expression level of *LGALS9* mRNA and its association with the clinicopathological characteristics of cervical squamous cell carcinoma samples. *LGALS9* expression was lower in advanced clinical stages (III and IV) (n=47) than in early clinical stages (I and II) (n=140). Student's t tests were used to determine the differences between groups, and $p < 0.05$ was considered significant. ** $p < 0.01$.

LGALS9 expression changes was also associated with clinicopathological characteristics in SCC; indeed, decreased *LGALS9* expression was associated to advanced clinical stages III and IV ($p = 0.0072$; Figure 3). There were no differences in other pathological characteristics analysed, such as differentiation degree, keratinization, and lympho-vascular invasion (data not shown).

Univariate and multivariate Cox regression analyses were performed to determine the association between overall survival and the *LGALS9* expression level, clinical stage, pathological grading, and keratinizing. Both the univariate and multivariate analyses showed a statistical association in the expression level of *LGALS9* and the clinical stage showing that they were independent prognostic factors (Table 2).

Enriched Pathways Associated to *LGALS9* Expression

GSEA was performed in samples with high and low *LGALS9* expression, and the analysis showed 7 pathways related to high *LGALS9* expression (Table 3). Some of them are involved in the immune response, such as complement, interferon_alpha_response, and other pathways are involved in cellular metabolism, such as xenobiotic_metabolism and bile_acid_metabolism. The results showed some of the biological pathways enriched with the high expression of *LGALS9* are implicated in the regulation of immune processes.

LGALS9 Expression is Associated with Abundance of Tumour Infiltrating Immune Cells

The correlation between *LGALS9* expression and the tumour infiltrating immune cells was determined with data of the TIMER database. Bioinformatic analysis showed that *LGALS9* expression was correlated with several immune cell infiltration. The strongest positive correlation was observed in neutrophils ($R = 0.406$, $p < 0.0001$); but there were slight

Table 2 Univariate and Multivariate Cox Regression Analysis of Clinical Stage, Pathological Grading, Keratinizing and *LGALS9* Expression Level

Variables	Univariate Model			Multivariate Model		
	HR	95% CI of HR	P value	HR	95% CI of HR	P value
Clinical stage						
I/II (n=135)	3.032	1.721–5.267	<0.0001	4.496	1.881–11.130	0.0008
III/IV (n=43)						
Pathological grading						
G1 y G2 (n=88)	0.810	0.411–1.531	0.5265	0.984	0.361–2.452	0.9743
G3 y G4 (n=73)						
Keratinization						
No-Keratinizing (n=74)	1.600	0.764–3.420	0.2144	0.808	0.316–2.031	0.6502
Keratinizing (n=37)						
LGALS9 expression level						
Low expression n=92	0.340	0.183–0.605	0.0004	0.379	0.165–0.828	0.0171
High expression n=91						

Notes: p<0.05 was considered statistically significant. The bold p values correspond to the variables with statistical significance.

Abbreviations: HR, hazard ratio; CI, confidence interval; n, number of samples.

Table 3 Gene Set Enrichment Analysis of High *LGALS9* Expression in Squamous Cervical Carcinoma Compared with Low *LGALS9* Expression

Name	Size	ES	NES	NOM p-val	FDR q-val
INTERFERON_ALPHA_RESPONSE	92	0.72	1.65	0.038	0.210
COMPLEMENT	195	0.52	1.61	0.027	0.174
PEROXISOME	101	0.36	1.57	0.022	0.186
APICAL_SURFACE	43	0.53	1.52	0.027	0.186
XENOBIOTIC_METABOLISM	196	0.41	1.48	0.027	0.187
BILE_ACID_METABOLISM	112	0.44	1.47	0.031	0.182
FATTY_ACID_METABOLISMO	153	0.34	1.47	0.039	0.171

Notes: p-val <0.05 and q-val <0.25 were considered significant. Size represents the number of genes altered in the pathological pathway.

Abbreviations: ES, enrichment score; NES, normalized enrichment score; NOM p-val, nominal p value; FDR, false discovery rate.

correlations in CD4+ T cells (R = 0.342, p < 0.0001), B cells (R = 0.286, p < 0.0001), CD8+ T cells (R = 0.229, p < 0.001) and dendritic cells infiltration (R = 0.198, p < 0.001), see [Table 4](#).

Galectin-9 Identification in Squamous Cervical Cancer Tissue

Galectin-9 expression level, the nuclear and cytoplasmic location in cervical cancer tissue varied between samples ([Figure 4A](#) and [B](#)). Some samples showed both cytoplasmic and nuclear expression ([Figure 4C](#)), others showed only a cytoplasmic location ([Figure 4B](#)), and some cellular nuclei showed high expression of galectin-9 ([Figure 4D](#)). The cellular localization was not related to the clinicopathological characteristics.

Table 4 Correlation Between the *LGALS9* Expression Level and the Abundance of Immune Cells Infiltrates in Cervical and Endocervical Cancer Tumours, Based on TIMER Database

Immune Cell Type	Cervical and Endocervical Cancer	
	Rho	P values
B Cell	0.28662951	1.23E-06
CD8 ⁺ T Cell	0.2297904	0.00012414
CD4 ⁺ T Cell	0.3423527	4.92E-09
Macrophage	0.10463008	0.08215953
Neutrophil	0.4065443	1.89E-12
Dendritic Cell	0.19854964	0.00091099

Based on the differences in the galectin-9 expression level between samples, we decided to determine whether the expression level was related to the clinicopathological characteristics. For the clinical stage characteristics, a significant decrease in galectin-9 expression was observed in advanced clinical stages ($p = 0.0336$) (Figure 5A). The analysis showed no relationship between the galectin-9 expression level and the degree of differentiation (G1/G2 vs G3/G4) and keratinization ($p = 0.2865$ and $p = 0.0671$, respectively) (Figure 5B and C).

The group of deceased patients showed lower galectin-9 expression in cancer tissue than the group of patients in remission of the disease ($p = 0.0487$), (Figure 5D).

Galectin-9 Expression Level in Cervical Squamous Cell Carcinoma and Its Association with Overall Survival

To determine if the galectin-9 expression level in SCC tissue was related to the overall survival of the patients, the samples were divided into two groups, high and low expression levels, considering the median of the group. After that,

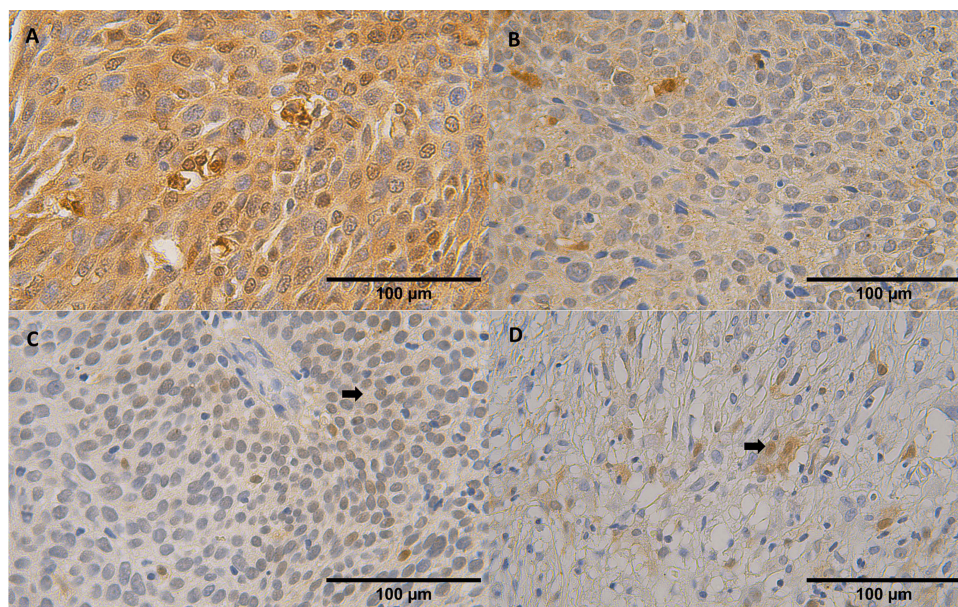


Figure 4 Galectin-9 expression and cell location in cervical squamous cell carcinoma tissue. (A) High galectin-9 expression in squamous cell carcinoma tissues. (B) Moderate galectin-9 expression in cervical cancer tissue. (C) Example of low galectin-9 expression, where the nuclear location of galectin-9 in cervical squamous cell carcinoma is indicated with an arrow. (D) Example of low galectin-9 expression, where the cytoplasmic location of galectin-9 in cervical squamous cell carcinoma is indicated with an arrow. Amplification 400x.

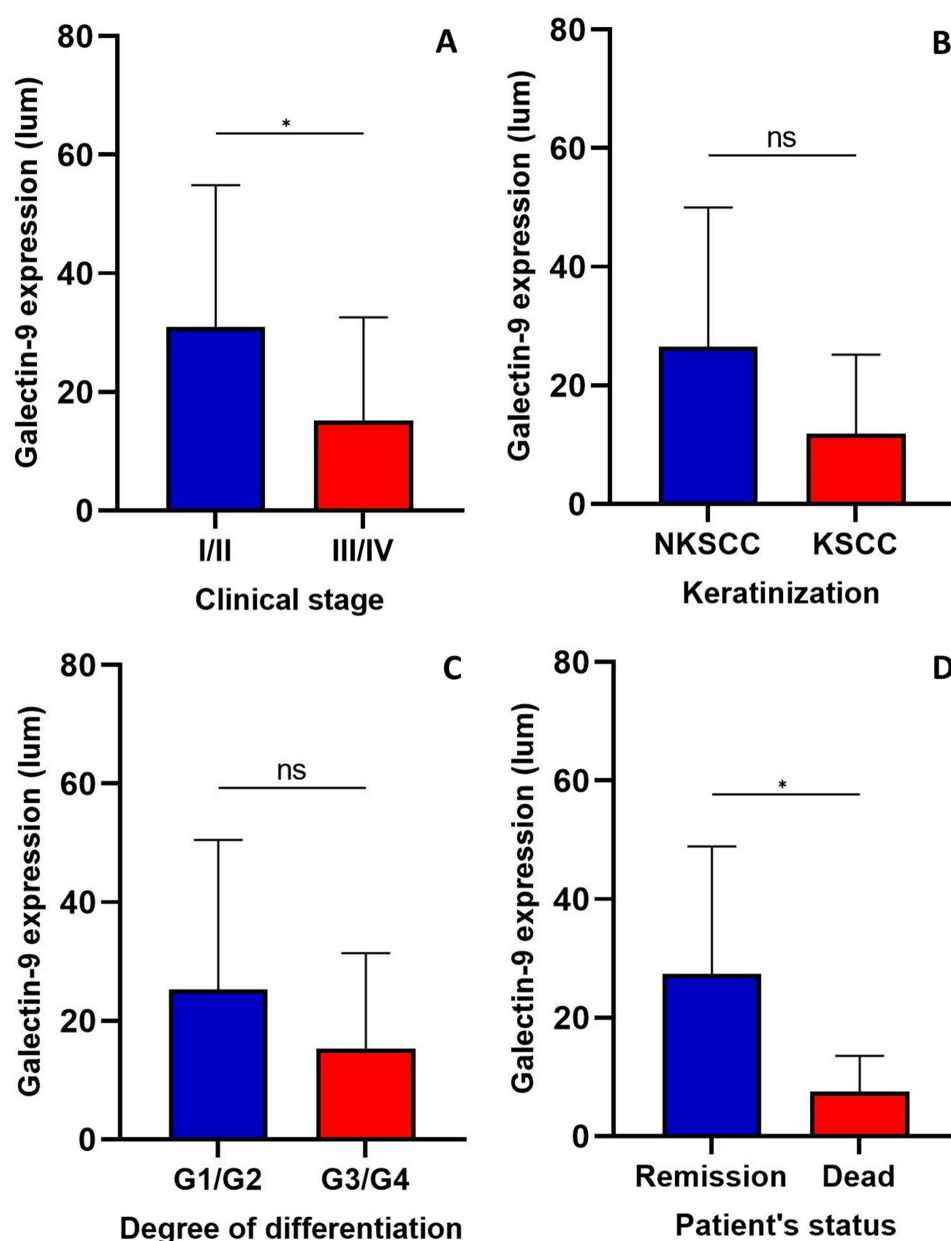


Figure 5 Galectin-9 expression level in cervical squamous cell carcinoma and its association with clinicopathological characteristics. **(A)** Galectin-9 expression is significantly higher in early clinical stages I and II ($n=19$) than in advanced clinical stages III and IV ($n=21$), with Mann–Whitney U -Test $p=0.0336$. **(B)** Galectin-9 expression level did not change with the grade of differentiation G1/G2 ($n=22$) vs G3/G4 ($n=13$), with Mann–Whitney U -Test $p=0.2865$. **(C)** Galectin-9 expression level did not change with the characteristic keratinizing ($n=8$) or nonkeratinizing ($n=27$), with Mann–Whitney U -Test $p=0.0671$. **(D)** Galectin-9 expression level was significantly higher in patients with remission ($n=8$) of the disease with respect to deceased ($n=6$) patients, with Student's T test $p=0.0487$. For all analyses, $p<0.05$ was considered significant. * $p<0.05$.

Abbreviation: ns, non-significant.

a Kaplan–Meier curve and a log rank analysis were performed. The results did not show a significant relationship ($p = 0.1378$); however, it is important to note that there was a tendency of better survival for the patients with higher expression of galectin-9 (Figure 6).

Discussion

Galectins play many different roles in cancer biology depending on their cellular or extracellular location, concentration, and the interaction with specific ligands in normal and immune cells.²⁴

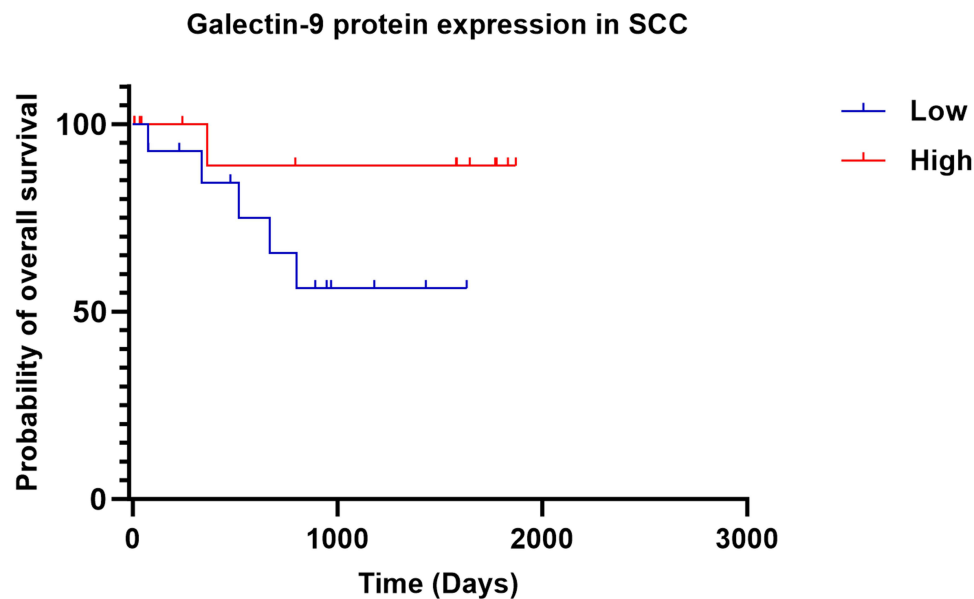


Figure 6 Overall survival of patients with squamous cell carcinoma in relation to high and low galectin-9 expression. The graphic shows a trend ($p=0.1378$) of better survival for the patients with high expression of galectin-9 ($n=14$) vs low galectin-9 expression ($n=14$). Kaplan–Meier curve and log rank analysis $p<0.5$ were considered significant.

Galectin genes can be up- or down-regulated in different cancer types, including cervical cancer, and have been involved in cancer progression.²⁵

In this study, we performed bioinformatic analysis to evaluate the expression of *LGALS* genes from a public database of cervical cancer and normal samples. Our results identified changes in the mRNA levels of *LGALS1*, *LGALS2*, *LGALS4*, *LGALS7* and *LGALS9*. Previous studies reported increased expression of Galectin-1 in cervical cancer tumours, but our results showed decreased levels of mRNA of *LGALS1*.¹⁰ *LGALS4* showed decreased expression in squamous cell carcinoma. Galectin-4 was evaluated by our research group in SCC, the expression level was higher in keratinizing tumours than in nonkeratinizing tumours, and a trend of higher expression was observed in tumours of clinically advanced stages.²⁶ The *LGALS7* gene showed increased expression in squamous cell carcinoma, but studies of galectin-7 in cervical cancer reported decreased expression, finding that tumours with high expression were related to a better prognosis.²⁷ Therefore, the mRNA levels of *LGALS1*, *LGALS4* and *LGALS7* genes was not related to protein expression, suggesting that a posttranscriptional mechanism could modulate protein synthesis.

Our results showed significant increased expression of *LGALS9* in both SCC and AC. In addition, patients with SCC and higher *LGALS9* expression showed a significant better survival, but not those with AC. Contrarily, Zhang et al, 2019, found that lower levels of *LGALS9* expression were associated with better overall survival.²⁸ However, their study included all the histological types in the correlation analysis; meanwhile our study analysed separately SCC and AC data, considering that they are tumours with different molecular characteristics.

In addition, we found association of higher expression of *LGALS9* with early clinical stages of SCC. Our findings of mRNA levels are supported with the expression of galectin-9 protein, Punt et al reported that cervical cancer tumours positive to galectin-9 were related with better survival.¹⁰

Additionally, enrichment analysis was performed to identify the pathways related to high expression of *LGALS9*. The results showed that galectin-9 in cervical cancer tumours is associated to the immune response pathways, as complement, and interferon_alpha_response, highlighting its relevance in the modulation of the tumour microenvironment.

The bioinformatic analysis showed that *LGALS9* expression was positively associated with immune cell infiltration, presenting from highest to lowest abundance: neutrophils, CD4+ T cells, B cells, CD8+ T cells and dendritic cells. Although this is an initial approach, the role of neutrophils in cancer development is of great interest because they are

included as a diagnostic marker in several types of cancer; indeed, in ex vivo assays it has been reported that galectin-9 activate neutrophils, and also increase neutrophils' life span,²⁹ other studies reported that galectin-9 activate neutrophil-mediated cytotoxic activity in cancer cells.³⁰ Additionally, it has been reported that galectin-9 could play an antitumor immunity activating antigen presentation by dendritic cells,³¹ although in our study dendritic cells infiltration showed the lowest correlation.

Galectin-9 expression was identified in both the nucleus and cytoplasm of tumour cells. Beyer reported galectin-9 location only in the cytoplasm of SCC and they found that higher cytoplasmic galectin-9 expression in tumour cells is related to better prognosis.²² In the samples analysed, we observed tumours with only a cytoplasmic location and others with both a nuclear and cytoplasmic location, but we did not found a relationship between the subcellular location and any clinicopathological characteristics.²² Changes in galectin subcellular location in cancer have been reported for other galectins. The expression of galectin-7 increases in oesophageal squamous cell carcinoma tumours with respect to normal tissue; but it was mainly observed in the nucleus of normal cells, meanwhile it was found in the cytoplasm, nuclei, and membranes of cancer cells.³² Galectin-3 also changed its localization in melanoma; indeed, higher expression of nuclear galectin-3 was associated to better survival than patients with lower nuclear galectin-3 expression.³³ The subcellular location of galectin-9 in cervical cancer tumours must be explored in future studies to determine a possible relationship with its functions.

In our study, we found higher galectin-9 expression in early clinical stages. Our results agree with a previous report that also found higher expression of galectin-9 in SCC tumours with lower FIGO status. Beyer et al reported higher expression levels in SCC and FIGO stage I, and they also reported higher expression of galectin-9 in lower grading tumours.²²

We also evaluated galectin-9 expression with respect to patients with disease remission vs deceased patients, and higher expression was observed in the first group. The analysis of overall survival and galectin-9 expression levels showed a trend towards better survival in patients with higher expression levels. Punt et al also reported a trend of better survival for patients with tumours with higher expression of galectin-9.¹⁰ This is the first study that identify in cervical cancer tumours with high expression of *LGALS9* the enrichment of genes related to immune pathways and major abundance on tumour infiltrating immune cells. The potential role of galectin-9 in the regulation of immune response in cervical cancer should be analysed in more detail in further studies.

Conclusion

Higher mRNA levels of *LGALS9* and galectin-9 correlated with early clinical stages and better survival in patients with cervical SCC. In addition, *LGALS9* levels were positively associated with immunological pathways and infiltration of tumour immune cells, highlighting the role of galectin-9 as an immunomodulator and a potential prognostic biomarker.

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Disclosure

The authors report no conflicts of interest in this work.

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