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ORIGINAL RESEARCH

Serum Levels of Galectin-9 are Increased in Cervical Cancer Patients and are Higher in Advanced Clinical Stages

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Purpose: Cervical cancer (CC) is the second most frequent cancer in undeveloped countries. Serum biomarkers could be useful for evaluation of the treatment response and as a complementary means to improve diagnosis. The expression of galectin-9 is altered in cancer tissue, and higher concentrations are found in the serum of cancer patients. The objectives of this study were (a) to determine the serum galectin-9 concentration in patients with intraepithelial lesions and CC, (b) to determine if the concentration was related to the clinicopathological characteristics and (c) to determine if the galectin-9 concentration was related to its expression level in tumour tissue.

Patients and Methods: In all, 222 serum samples from women with different diagnoses, including premalignant lesions and CC, as well as samples from women with normal cytology were included in the study. The serum galectin-9 concentration was determined by ELISA. To evaluate the expression level of galectin-9 in CC tissue, immunohistochemistry was performed in 34 CC biopsy specimens.

Results: The galectin-9 concentration in the serum of CC patients (8.171 ng/mL) was increased compared with serum from women with normal epithelia (4.654 ng/mL) and those with low-grade (4.806 ng/mL) and high-grade (5.354 ng/mL) intraepithelial lesions (p value < 0.0001). The area under the ROC curve considering the CC group and the control group was 0.882. The optimal cut-off value was ≥6.88 ng/mL, the specificity obtained was 100%, and the sensitivity was 68.2%. In the CC group, the analysis of the clinical stage showed an increase of galectin-9 in the advanced stage IV group. Serum galectin-9 was not related to the level of galectin-9 expression in tissue, which suggests that galectin-9 is not secreted by tumour cells.

Conclusion: The serum galectin-9 concentration is related to cancer progression, as the level of this protein is higher in patients with advanced-stage disease.

Keywords: galectin-9, serum biomarker, prognostic markers, cervical cancer, premalignant lesions

Introduction

Cervical cancer (CC) is the fourth most common cancer in females worldwide and is the second most deadly cancer in women in developing countries.[1] In Mexico, the lack of regular screenings and the high percentage of false-negatives of the Pap smear test have resulted in late disease diagnosis.[2,3] The Thin Prep Pap Test and human papillomavirus detection have improved diagnostic sensitivity, but these are not used in national screening programs and are not accessible to most of the population.[4] Therefore, novel biomarkers could help in early CC diagnosis, evaluation of the treatment response and disease prognosis.[5] In recent years,

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galectins have become of great interest in cancer research due to their participation in tumour progression.[6] Galectins are a family of carbohydrate-binding proteins with special affinity for β-galactosides.[7] Galectin-9 is a tandem repeat protein with two carbohydrate recognition domains (CRDs) connected by a linker peptide.[7,8] As with other galectins, galectin-9 can regulate a variety of biological functions, such as cell aggregation, migration, and apoptosis.[8–10] Different studies have reported that galectin-9 negatively regulates the immune response and plays an immunosuppressive role. Galectin-9 induces T-cell death through its interaction with Tim-3. The activation of Tim-3/galectin-9 pathways in cervical cancer patients negatively regulates the cellular immune response. In vitro assays showed that blocking this pathway rescued T-cell proliferation.[11] Galectin-9 can also interact with the innate immune receptor dectin-1, which induces the polarization of macrophages to the M2 phenotype.[12] Galectin-9 tissue expression has been reported to be altered in gastric cancer, breast cancer and non-small cell lung cancer, among others.[13–15] The serum galectin-9 concentration is increased in different cancer types, including pancreatic ductal adenocarcinoma, chronic lymphocytic leukaemia and cutaneous T-cell lymphoma.[16–18]

Galectin-9 expression in cervical intraepithelial neoplasia and cervical squamous cell carcinoma (SCC) is reduced compared with normal epithelial tissue.[19] Additionally, patients with galectin-9-positive tumour cells show a trend towards improved survival.[20,21] Galectin-9 has been implicated in tumorigenesis and is a potential prognostic marker in CC.[22]

Only one study has been published on the serum concentration of galectin-9 in CC, and in that study, the concentration was reported to be increased.[23] Therefore, the aims of this study were to determine the concentration of serum galectin-9 in patients with low- and high-grade squamous intraepithelial lesions and CC to determine whether the clinicopathological characteristics were related to changes in galectin-9 and whether the serum concentration of this protein was related to galectin-9 tissue expression.

Materials and Methods

Patients and Samples

This study was conducted in accordance with the Declaration of Helsinki, and the ethical regulations were approved by the Human Ethics and Research Committee number 785 from the Instituto Mexicano del Seguro Social (IMSS). The registration number of the project is R-2017-785-119. All women included in the study were informed and signed a consent form before sample collection.

Women whose cytology reports were negative for intraepithelial lesions or malignancy (control group), and those with low-grade squamous intraepithelial lesions (LSIL), high-grade squamous intraepithelial lesions (HSIL), and CC were included in the study. Cases were diagnosed by a pathologist according to the Bethesda System. The clinical stages of patients in the CC group were determined according to the FIGO staging system.

Serum was obtained from women with CC when they presented to the Radiotherapy Service of the National Health Centre, Manuel Avila Camacho, IMSS, in Puebla City from February 2017 to December 2020. Patients had not received any previous treatment. Women who were previously diagnosed with another cancer type, were pregnant, or had acute infections were excluded. Serum samples from women whose cytology reports were negative for intraepithelial lesions or malignancy and samples from women diagnosed with intraepithelial lesions were obtained from the Clinic of Dysplasia at the Zone General Hospital Number 5, IMSS, in Metepec, Puebla. Serum was obtained by phlebotomy and was maintained at −20°C until use. CC biopsies were obtained from the Pathology Service of the same hospital, biopsies were evaluated by the pathologist according to Bethesda diagnostic criteria. The clinicopathological data of the CC group were obtained from clinical records.

Serum Galectin-9 Concentration

The serum galectin-9 concentration was determined using a Quantikine ELISA Human Galectin-9 Kit (DGAL90 from R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions. Duplicates of serum samples were used at a dilution of 1:2. Plates were read in a Biotek Synergy-4 plate reader (Winooski, VT, USA) at 450 nm and 570 nm. To correct the optical imperfections of the plate, the values obtained from the readings at 450 nm were subtracted from the readings at 570 nm. An average of the duplicates of each sample was obtained, and the serum galectin-9 concentration determined by a standard curve was multiplied by the dilution factor.

Tissue Galectin-9 Expression

Paraffin blocks were sectioned (5 µm) and placed on poly-L-lysine-coated glass slides. The sections were deparaffinized for 1 h at 60°C. To block the activity of endogenous peroxidase, the sections were incubated in methanol containing 0.3% H2O2 for 30 min. Thereafter, every step was followed by three washes in PBS buffer. To block nonspecific antibody binding, slides were incubated for 1 h in 1% BSA in PBS. Sections were incubated with galectin-9 antibody (ab69630, Abcam, Cambridge, MA USA) diluted at 1:400 overnight at 4°C. Then, the sections were incubated with HRP-conjugated anti-rabbit IgG (ab6721, Abcam) for 1 h at room temperature. Staining was visualised by incubating the slides with DAB substrate (ImmPACT DAB Peroxidase substrate, Vector SK-4105, Burlingame, CA USA) according to the manufacturer's instructions. To distinguish the nuclei, sections were counterstained in haematoxylin solution (HX87960674 Merck, Darmstadt Germany) for 10 sec. Images were obtained using a VENTANA DP200 scanner (Roche Diagnostics, Tucson, AZ USA). The intensity of galectin-9 expression was determined using Image-Pro Image Analysis Software (Media Cybernetics, Rockville, MD USA). The mean density was calculated for each sample and the expression of galectin-9 was reported in lumens.

Statistical Analysis

To assess the difference and correlation between a set of nonparametric data, the Kruskal–Wallis test followed by Dunn's multiple comparison test was performed, except when analysing keratinization, where the Mann–Whitney *U*-test was used. Additionally, correlation analyses were performed using Spearman's rank correlation coefficient. All statistical analyses were performed using Prism 5 software (GraphPad software, La Jolla, CA, USA). $p \leq 0.05$ was considered significant. To determine the diagnostic value of serum galectin-9 to discriminate between controls, LSIL, HSIL and CC patients, receiver operating characteristic (ROC) curves were generated using SPSS statistical software version 26 (IBM; Armonk, NY, USA).

Results

Serum Levels of Galectin-9

In all, 222 women were included in the study, and of these, 34 had a negative pap result for neoplasia or malignancy, 27 had LSIL, 29 had HSIL and 132 had CC.

The serum concentration of galectin-9 was determined by ELISA for the control, LSIL, HSIL and CC groups. The results showed that galectin-9 was significantly higher in the CC group (8.171 ng/mL) than in the HSIL (5.354 ng/mL), LSIL (4.806 ng/mL) and control (4.654 ng/mL) groups (Figure 1). Some CC patients showed a concentration 6 times higher than the mean concentration of the control group.

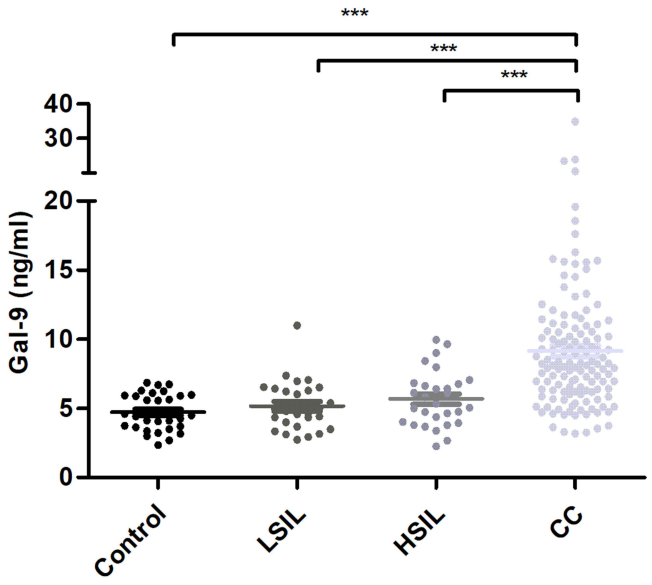


Figure 1 Galectin-9 serum concentration is shown for the control, LSIL, HSIL, and CC groups. The concentration was higher in the CC group. Each point corresponds to data from one patient. Kruskal–Wallis *** $p < 0.0001$.

Relationship Between Serum Levels in the CC Group and Clinicopathological Characteristics

The clinicopathological features of the CC group are shown in Table 1. We determined whether the serum levels of galectin-9 were related to the clinicopathological characteristics of CC. The results showed that the serum concentration of galectin-9 was not related to histological type, differentiation grade or whether the tumour was keratinizing or nonkeratinizing. The serum concentration of the clinical stage IV (10.80 ng/mL) group was significantly higher than that of the stage I (6.851 ng/mL) and II (7.805 ng/mL) groups (Figure 2).

ROC Curve Analysis and Youden Index

To determine the diagnostic potential of the serum concentration of galectin-9, we performed a ROC curve analysis and compared the CC group to the control group and the HSIL and LSIL groups to the control group. The area under the curve (AUC) for the analysis of the CC group was 0.882, and thus, galectin-9 is a good discriminator of cervical cancer patients. The Youden index and the optimal cut-off values were 0.682 and ≥6.88 ng/mL, respectively. The specificity obtained was 100%, and the sensitivity was 68.2% (Figure 3). The AUC of the ROC curves for the HSIL and LSIL groups was 0.642 and 0.561, respectively, which shows that galectin-9 is a poor discriminator. The HSIL and CC groups and the LSIL and CC groups were also compared. The area under the curve for the LSIL and HSIL groups versus CC was 0.776 and 0.835, respectively, and thus, galectin-9 is a good discriminator of patients with LSIL and a moderate discriminator of patients with HSIL compared with CC patients (Figure 4).

Correlation Between Galectin-9 Serum Levels and Tissue Expression

To determine whether the tumour expression level of galectin-9 was related to the serum concentration, we compared the serum concentration and tumour expression in samples from the same patient. To achieve this, 34 CC biopsies were stained using immunohistochemistry, the expression level was determined using Image-Pro Image Analysis Software, and it was reported in lumens. The expression level of galectin-9 was different between specimens (Figure 5), the Table 2 shows the expression level of galectin-9 in lumens for samples of each histological type and clinical stage. Spearman correlation was performed to compare the expression level in the biopsy tissues and the corresponding serum concentration. The results showed that the serum concentration of galectin-9 was not related to the expression level in the tumour ($r = 0.05302$, $p = 0.7587$) (Figure 6).

Table 1 Clinicopathological Characteristics of the CC Group

Histological Type			
Squamous Cell Carcinoma	**Adenocarcinoma**		**Adenosquamous**
107	16		7
Differentiation grade			
Well	Moderate	Poor	Non-differentiated
7	69	29	7
Keratinizing			
Yes		No	
21		67	
FIGO staging			
Stage I	Stage II	Stage III	Stage IV
14	42	43	17

Note: the number of samples is shown for each group.

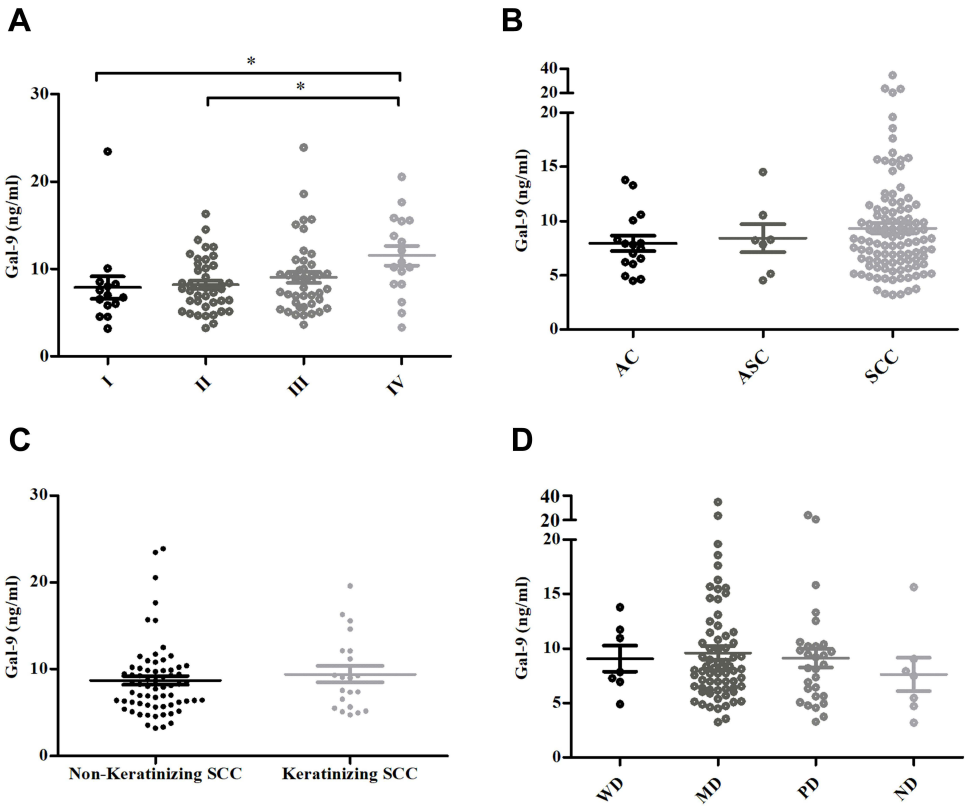


Figure 2 Serum galectin-9 concentration and the clinicopathological characteristics. (**A**) Serum concentration among the different clinical stage groups. (**B**) Serum concentration among the histological types: squamous cell carcinoma (SCC), adenosquamous carcinoma (ASC), adenocarcinoma (AC). (**C**) Serum concentration of galectin-9 in the keratinizing and nonkeratinizing tumour groups. (**D**) Serum concentration among the differentiation grades (well, moderately, and poorly differentiated and undifferentiated). Each point corresponds to data from one patient. Kruskal–Wallis * p< 0.05.

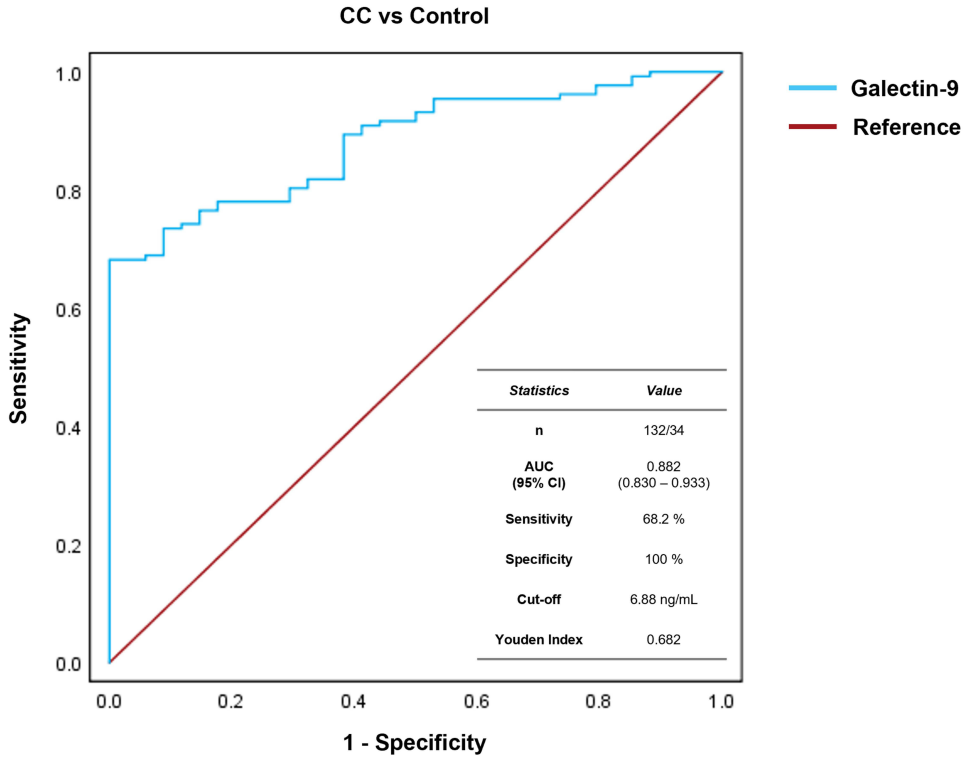
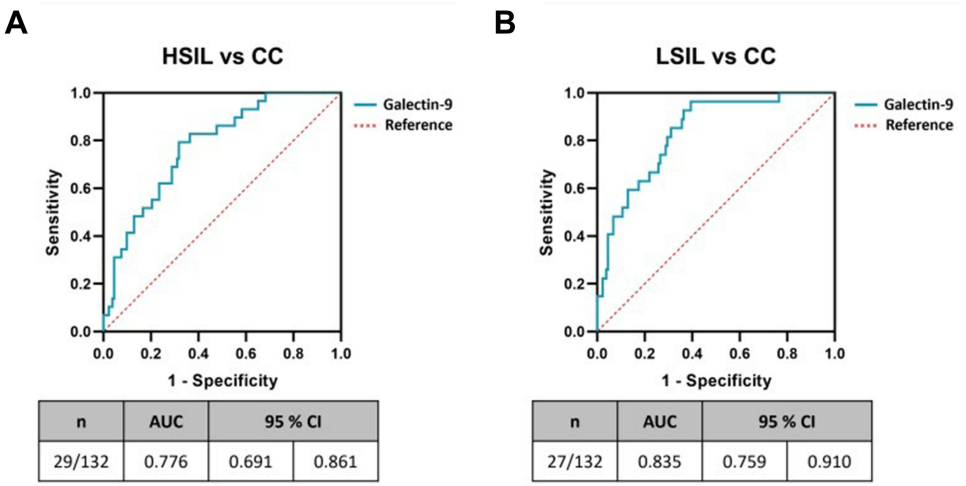


Figure 3 ROC curve for serum galectin-9 concentration in patients with CC versus controls.



n	AUC	95 % CI	
29/132	0.776	0.691	0.861

n	AUC	95 % CI	
27/132	0.835	0.759	0.910

Figure 4 ROC curve analysis of serum galectin-9 in patients with HSIL or LSIL versus CC.

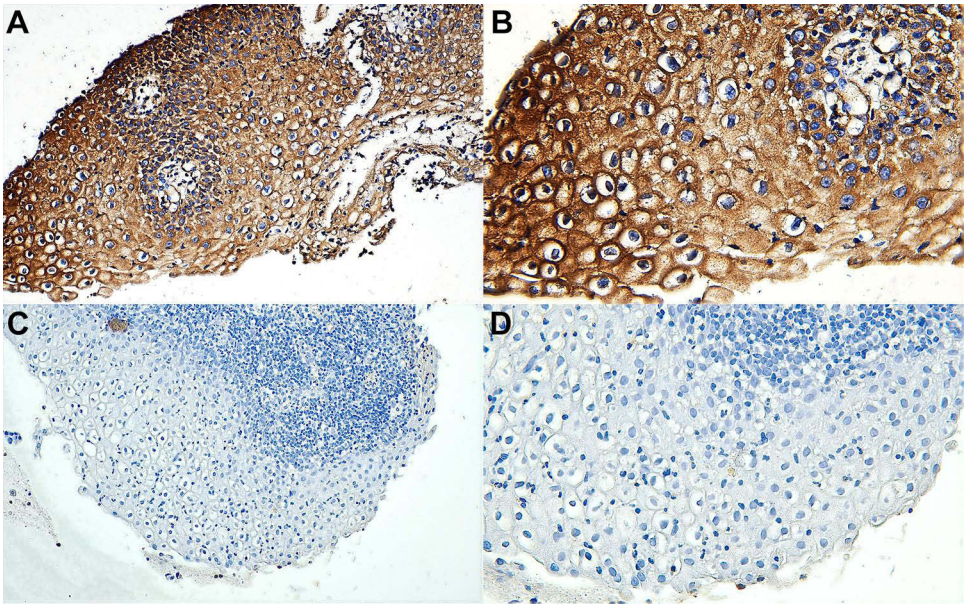


Figure 5 Expression of galectin-9 in CC tissue. CC specimen with high expression (**A**) 10X amplification, (**B**) 20X amplification. CC tissue with low galectin-9 expression (**C**) 10X amplification and (**D**) 20X amplification.

Discussion

Altered expression of galectin-9 has been reported in the tumours of different cancer types and has been associated with the disease prognosis.[13–15] Circulating galectin-9 has also been reported in some cancer types, in patients with advanced melanoma the plasma concentration is higher compared to controls and has been related with reduced survival.[24]

In our study, a higher serum galectin-9 concentration was detected in CC patients compared with control, LSIL and HSIL patients. The results agree with a previous report that found higher galectin-9 concentrations in patients with CC compared with patients with cervical intraepithelial neoplasms and patients with normal cytology.[23]

Increased concentrations have also been reported in patients with pancreatic ductal adenocarcinoma (PDAC), in which the Galectin-9 serum concentration was able to discriminate PDAC patients from patients with benign pancreatic disease and healthy controls.[16] Our results did not show a significant difference between the premalignant and control groups, which suggests that serum galectin-9 should not be used for early diagnosis.

Table 2 Expression Levels of Galectin-9 in CC Tissue According to Histological Type and FIGO Staging

Histological Type				
	Squamous Cell Carcinoma	**Adenocarcinoma**		**Adenosquamous**
Number of samples	27	4		3
Median (lum)	32.82	79.18		48.57
Range	(0.3–138.08)	(0.50–91.73)		(0.10–91.73)
FIGO staging				
	Stage I	Stage II	Stage III	Stage IV
Number of samples	4	17	10	4
Median (lum)	48.51	53.77	39.37	34.22

Note: 1 sample has no FIGO classification.

ROC curve analysis was performed to determine the diagnostic value of serum galectin-9 to distinguish the control and CC groups. The results showed that this protein is a good discriminator of CC patients. Additionally, considering the cut-off value, the specificity obtained was 100%, and no false-positives were detected; however, some women with CC were not detected (sensitivity 68.2%). Therefore, the use of serum galectin-9 as a diagnostic tool for CC could result in a significant number of false-negatives. The same problem has been reported for Pap smears. A study that evaluated the quality of Pap readings in several cytology laboratories in Mexico showed that some of tests had a sensitivity less than 65%; this demonstrates a lack of reproducibility related to high rates of false-negatives, which has an impact on higher disease incidence.[3] The search for detection methods that improve diagnostic sensitivity in CC has gained relevance. Although the sensitivity of serum galectin-9 for the diagnosis of CC is moderate, the specificity is high, and it could be used as a complementary approach. Therefore, the application of both the Pap smear and serum galectin-9 tests could improve the sensitivity for detecting CC patients, but this combination must be evaluated. Galectin-9 was shown to be a poor diagnostic discriminator of patients with HSIL and LSIL, and thus, serum galectin-9 seems to be relevant only for CC patients; however, we cannot rule out the possibility that galectin-9 plays a role in the persistence of premalignant lesions, but this must be analysed. Furthermore, our results showed that the galectin-9 concentration did not change in

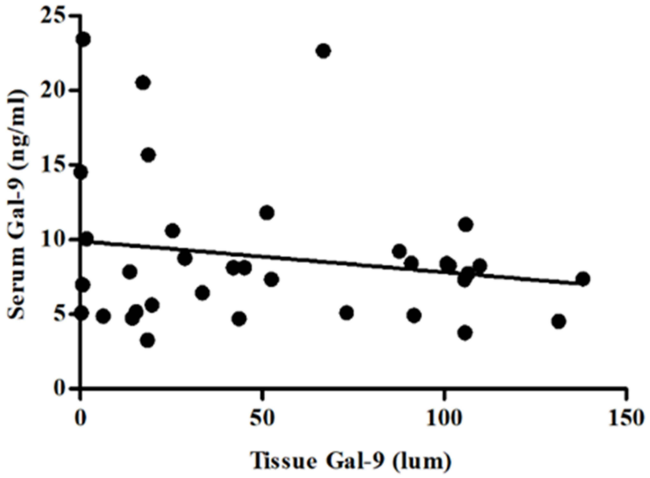


Figure 6 Correlation of galectin-9 in serum and tissue. No correlation was found between serum galectin-9 and its expression in tissue. Spearman correlation test, r=0.05302.

relation to the tumour characteristics, such as histological type, differentiation grade, and keratinizing or nonkeratinizing type, but its concentration was increased in advanced FIGO stages; thus, galectin-9 could be an indicator of disease progression. This finding has not been previously reported in CC patients. An increased concentration of serum galectin-9 has been previously related to disease stage in patients with chronic lymphocytic leukaemia, where patients with Binet stages B and C exhibited higher levels of galectin-9 than patients with Binet stage A.[17] It is known that galectin-9 is a ligand for Tim-3, and it has been reported that through its interaction with Tim-3, galectin-9 can induce CD4+ T-cell apoptosis.[25] Additionally, the galectin-9/Tim-3 interaction inhibits the activation of NK cells in acute myeloid leukaemia.[26] In osteosarcoma, signalling between galectin-9 and Tim-3 is associated with suppression of the Th1 response.[27] This suggests that serum galectin-9 may participate in the inhibition of the antitumor immune response in CC, thus promoting cancer progression, which would be related to the higher levels observed in advanced stages.

In 2008, Liang et al suggested that serum galectin-9 could be secreted by cervical tumours because they reported two patients with higher levels of serum galectin-9 and intense staining in the tumour tissue.[23] Therefore, we evaluated a higher number of patients, but we did not find a relationship between galectin-9 tissue expression and serum concentration, which supports the idea that serum galectin-9 is not secreted by tumour cells. In 2021, Chen et al reported that galectin-9 expression in monocytes of patients with HPV-positive CC is higher than that in patients with HPV-negative CC or benign uterine fibroids. Their results suggest that the high levels of galectin-9 in monocytes of HPV-positive CC patients could be related to disease progression; these findings support the importance of galectin-9 in CC.[11] Considering the increase expression of galectin-9 on monocytes of CC patients and on blood γδ T cells of patients with pancreatic ductal adenocarcinoma, it has been proposed that serum galectin-9 could be secreted by blood immune cells, but this finding requires confirmation.[11,16]

The immunomodulatory role of galectin-9 indicates that it is involved in pathologies other than cancer. Serum galectin-9 has been reported to be increased in some autoimmune and infectious diseases, such as lupus erythematosus, atopic dermatitis, and systemic sclerosis, and in some infectious diseases, such as dengue and chikungunya. The role of galectin-9 in the modulation of the immune response seems to be relevant in these diseases but also in cancer.[28–31] Galectin-9 has been proposed as a therapeutic target for cancer, as this protein induce T-cell death and immunosuppression in the tumour microenvironment.[32]

The transcriptional regulation of galectin-9 gene (*LGALS9*) can be modulated by epigenetic mechanisms, our research group have found in cervical cell lines that *LGALS9* expression level is positively related to the H3K9 and H3K14 histone acetylation and Zhang et al in 2019 found that *LGALS9* is negatively regulated by an epigenetic mechanism related to the infection of HPV-18. It has been reported that DNMT3A contributes to the transcriptional silencing of *LGALS9* by the promoter methylation.[33–35]

Many questions still exist about the origin and functions of serum galectin-9 and its role in cervical cancer progression, but also about the mechanisms involved in the deregulation of its expression.

Conclusion

Our results provide new information on serum galectin-9 in CC patients. Serum galectin-9 is increased in patients with CC, and the AUC of the ROC analysis supports the finding that serum galectin-9 is a good discriminator of CC patients. Considering the immunosuppressive role of serum galectin-9, it may be important to analyse this protein in future studies to determine whether the serum concentration of galectin-9 is higher in patients with persistent cervical lesions or in CC patients who do not respond to treatment. In addition, higher concentrations of galectin-9 were detected in patients with clinical stage IV disease, which indicates that this protein could be involved in disease progression and could be used as a prognostic biomarker and as a therapeutic target. The origin of circulating galectin-9 is uncertain, but our results support the idea that this protein is not secreted by tumour cells.

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Disclosure

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