Hindawi
BioMed Research International
Volume 2020, Article ID 6756723, 9 pages
https://doi.org/10.1155/2020/6756723

Research Article

Evaluation of Serum Levels and Expression of Galectin-4 in Cervical Cancer

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Received 16 July 2020; Revised 6 November 2020; Accepted 10 November 2020; Published 20 November 2020

Academic Editor: Rafael S. De Molon

Galectin-4 has been reported to be altered in different cancer types. Its expression changes have been associated with early recurrence and metastasis. In cervical cancer (CC), galectin-4 has not been studied. The aim of the study was to determine the expression level and subcellular localization of galectin-4 in CC tissue and the concentration in the serum of patients with CC. For the analysis of serum levels of galectin-4, an ELISA assay was performed. To assess the expression in cervical tissue, immunohistochemical staining was performed. The results showed that the concentration of galectin-4 in the serum of patients with CC was higher (647.9 pg/ml) than that in the serum of women with normal cytology (382.1 pg/ml). The immunohistochemical analysis of CC samples showed a higher expression in keratinizing tumor than nonkeratinizing tumors and a trend of increased expression in tumors from patients with advanced clinical stage. In normal cervical tissue, galectin-4 was detected in the cytoplasm, and in tumor cells, the presence of galectin-4 was also detected in the nucleus, in both adenocarcinoma and squamous cervical cancer. The increase in serum concentration and different localization in the tumor cells suggest a possible role of galectin-4 in CC development.

1. Introduction

Cervical cancer (CC) is the second leading cancer-related cause of death in women in undeveloped countries [1]. The most common histological types are squamous carcinoma and adenocarcinoma [2]. CC screening is based on the test Pap smear (cytology), which has decreased the incidence and mortality of CC, but the low reproducibility of this test can impact the incidence rate; in México, a high percentage of false-negative cases have been reported [3]. Novel

biomarkers could help with early diagnosis, determine prognosis, evaluate the response to treatment, and clinically manage patients and therapies. The galectin family has 15 members that recognize β-galactoside structures through their carbohydrate-recognizing domains [4]. These proteins have been reported to be altered in different cancer types and, in some cases, associated with tumor progression and survival and have been proposed as biomarkers for prognosis and therapeutic targets [5–9]. For some galectins, changes in subcellular localization have been detected and associated with cancer progression, such as in tongue carcinoma, in which nuclear galectin-3 decreases with cancer progression, showing an increase in the cytoplasm [10]. The location of galectin-3 in the nuclei of non-small-cell lung cancer cells is a prognostic predictor of recurrence [11]. In oesophageal squamous cell carcinoma, high expression of galectin-7 has been reported with respect to normal tissue; additionally, galectin-7 was preferentially located in the nucleus of the cells in normal tissue, and in cancer tissue, it was located in in the cytoplasm and in the cell membrane in addition to the nucleus [12]. It has been reported that the subcellular location determines the role of galectins [13, 14].

Galectin-4 is a protein with two carbohydrate domains that have been reported to be altered in pancreatic cancer [15], gastric adenocarcinoma [16, 17], hepatocellular carcinoma [6], colorectal cancer, breast carcinoma [18], and lung cancer [19]. The role of galectin-4 can be different with respect to the cancer type. In pancreatic cells, it can decrease migration and metastasis [20]; in contrast, in colorectal cancer cells, the low expression of galectin-4 increases migration and cell proliferation [21]. The serum levels have also been reported to vary in different types of cancer such as hepatocellular carcinoma, melanoma, pancreatic cancer, bladder cancer, colorectal cancer, breast cancer, gastric cancer, and head and neck cancer, in which the concentration has been reported to be increased compared with that in normal tissues [22–27]. Serum galectins can promote migration and metastasis in cancer [28, 29].

For CC, neither the tissue expression nor the serum concentration of galectin-4 has been reported. Considering the important roles that this protein plays in the cell and its association with tumor progression, we were interested in evaluating the expression level of galectin-4 in CC tissue, its subcellular location, and its concentration in the serum of patients with CC.

2. Materials and Methods

2.1. Patients and Samples. The study was conducted in accordance with the Declaration of Helsinki and the ethical regulations approved by the Human Ethics Committee 2106 from the Instituto Mexicano del Seguro Social (IMSS), registration number (R-2016-2106-1).

The female patients included in the study were invited to participate at the Radiotherapy Service of the National Health Centre, Manuel Avila Camacho, IMSS, in Puebla City, Mexico, during the period February 2017 to July 2019. All the patients included in the study were informed and signed the consent form.

Clinical and pathological diagnoses of CC were made by the Pathology Service and treating oncologists according to FIGO staging [2].

2.2. Galectin-4 Detection in the Serum of Patients. A total of 52 female serum samples were collected, including 13 with normal cytology and 39 patients with CC. Serum was obtained by phlebotomy of newly diagnosed patients before treatment. The concentration of galectin-4 in serum was determined by using the Human Galectin-4 ELISA Kit (IRK-TAH5118, Innovative Research, Inc., Plymouth, MN, US) according to the manufacturer's instructions. Serum samples were used at a dilution 1 : 2.

2.3. Galectin-4 Detection in Cervical Tissue. Paraffin-embedded cervix biopsies were obtained from the Department of Pathology of the Centro Médico Nacional, Manuel Ávila Camacho, IMSS. A total of 35 biopsies were obtained, 6 from normal cervix tissue and 29 from patients with a diagnosis of CC. Paraffin sections 4 μm thick were cut and placed on slides treated with APES; next, they were deparaffinized and rehydrated. Antigen retrieval was performed by incubation for 15 min at 90°C in 10 mM citrate buffer pH 6.0 (C9999, Sigma-Aldrich, St. Louis, MO, US). Then, every step was followed by washing three times with 1x PBS except incubation with blocking buffer. To block endogenous peroxidase activity, sections were incubated for 15 min with 0.3% H_2O_2 in 1x PBS. Afterward, the tissues were incubated for 1 hour with blocking buffer containing 5% bovine serum albumin (P6154, Biowest, Riverside, MO, US.) in 1x PBS. The tissues were incubated overnight at 4°C with rabbit polyclonal anti-GAL4 antibody (ab170638, Abcam, Cambridge, UK) at a dilution of 1 : 100. Next, the tissues were incubated with the secondary antibody anti-rabbit IgG-HPR (ab6721, Abcam, Cambridge, UK) at a dilution of 1 : 1,000, and colour development was assessed with ImmPACT® DAB Peroxidase Substrate (SK-4105, Vector Labs, Burlingame, CA, US). Finally, nuclei were counterstained with haematoxylin solution (HX87960674 Merck, Darmstadt, Hesse, DE), and the slides were mounted with VectaMount® Permanent Mounting Medium (H-5000, Vector Labs, Burlingame, CA, UU). Negative controls of the technique were processed with only the secondary antibody.

Tissue images were obtained with a VENTANA DP200 scanner (Roche Diagnostics, Switzerland, CH), and the mean staining density was evaluated with the Image-Pro software (Media Cybernetics, Inc., Rockville, MD, US).

2.4. Statistical Analysis. Statistical analysis was performed using GraphPad Prism 8 (GraphPad Software, San Diego, CA, US). Statistical significance in serum was evaluated with the Mann–Whitney test. The association between the concentration of galectin-4 in serum and clinicopathological features was evaluated with a Mann–Whitney test with the exception of the differentiation grade which was calculated using Kruskal-Wallis test. The differences in staining intensity of the tissues between different stages of cancer were evaluated by the Mann–Whitney test. A value of $p < 0.05$ was considered to indicate a statistically significant difference.

3. Results

3.1. Serum Levels of Galectin-4 Are Higher in Patients with CC than in Patients without Disease. Serum levels of galectin-4 were evaluated by ELISA in 52 serum samples, including samples classified according to histological diagnosis as nondiseased ($n = 13$) and as CC ($n = 39$). The mean age of nondiseased patients was 31.72 years (±8.73 (SD); range 22-51 years), and for patients with a diagnosis of CC, it was 54.23 years (±14-24; range 21-81 years). The CC group comprised 32 patients with a diagnosis of squamous carcinoma and 7 patients with adenocarcinoma.

Patients with CC showed an increase in the serum concentration of galectin-4 with respect to nondisease patients. The median galectin-4 concentration was 382.1 pg/ml in nondisease patients and 647.9 pg/ml ($p = 0.0061$) in CC patients. The results are shown in Figure 1.

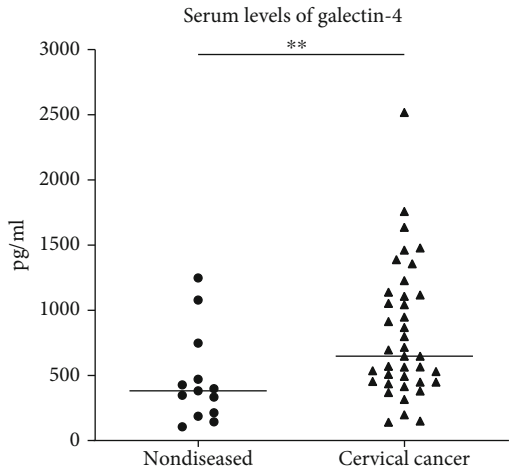

Figure 1: The concentration of galectin-4 was increased in serum from CC patients. Galectin-4 concentrations (pg/ml) in the serum of women without disease (circle, $n = 13$) and with CC (triangle, $n = 39$) are shown. Mann–Whitney test $^{**}p = 0.0061$.

To determine whether the serum concentration of galectin-4 in CC patients was related to clinicopathological features, histological type, differentiation grade, FIGO staging, tumor size, or keratinizing or not keratinizing type, a Mann–Whitney test was performed. The serum concentration did not show significant differences according to the analysed characteristics (Table 1). However, it is important to note that there was a tendency of increasing concentration in the group with keratinizing tumors with respect to the nonkeratinizing group. The concentration in the serum of patients with advanced clinical stages III-IV was also observed to be higher than that in the serum of patients with clinical stages I and II, who showed the lowest serum concentrations.

3.2. Galectin-4 Expression in Squamous Cervical Tissue. The expression of galectin-4 was evaluated by histochemical analysis in normal cervical epithelium to determine if this protein was expressed in the cervix and to subsequently evaluate its expression in CC tissues. The analysis was performed on 6 normal samples and 29 CC samples (23 with a diagnosis of squamous cervical carcinoma, 5 with a diagnosis of adenocarcinoma, and 1 with a diagnosis of adenosquamous carcinoma).

The presence of galectin-4 was detected both in normal tissue and in CC tissues. In the basal cells of the normal cervical squamous epithelium, galectin-4 was not detected, but it was observed in the differentiated cells throughout the layers of the epithelium. Regarding the cellular localization, galectin-4 was preferentially observed in the cytoplasm (Figure 2). Additionally, we observed the presence of galectin-4 in stromal cells.

In squamous cervical carcinoma, the expression of galectin-4 was detected in the tumor cells, but the expression pattern was different from that in normal tissue. In the tumor cells, the presence of galectin-4 was observed in both nuclei and the cytoplasm, and in some samples, the nuclear expression was higher than that in the cytoplasm (Figure 2). In the tumors, the expression of galectin-4 in stromal cells was also detected.

The expression level of galectin-4 between the tumors of patients with different clinical stages is shown in Figure 3. The level of expression and the cellular localization of galectin-4 did not change with clinical stage, and there were samples with different clinical stages that presented similar expression levels. This was also observed in relation to nuclear localization, for which the staining pattern did not change with cancer progression.

3.3. Galectin-4 Expression in Columnar Cervical Epithelium. Only a few samples of columnar epithelium and adenocarcinoma could be analysed, including 2 normal tissues and 5 adenocarcinomas, but we could observe characteristic expression patterns. In the columnar cells of the normal glandular epithelium, galectin-4 was detected at low intensity, and its expression was observed in the cytoplasm (Figure 4). When galectin-4 was analysed in the tumor specimens, the expression of galectin-4 was increased in the columnar cells, in which it was detected in nuclei (Figure 4).

3.4. Expression Level of Galectin-4 in Cervical Squamous Carcinoma. The expression level of galectin-4 in CC tissues was evaluated with respect to clinical stage, tumor differentiation grade, and keratinization status. The keratinizing tumors showed increased expression of galectin-4 with respect to the nonkeratinizing tumors, and the differences were statistically significant (Figure 5).

The statistical analysis did not show differences with respect to clinical stage and differentiation grade, but a trend of increasing expression was observed in the tumors of patients with advanced clinical stages (III and IV) (Table 2). It was also observed that most of the samples with the lowest expression of galectin-4 belonged to clinical stages I and II (Figure 6).

4. Discussion

The aim of this study was to determine whether the serum concentration of galectin-4 in patients with CC is different from that in healthy women and to evaluate the expression level and localization of galectin-4 in CC tissue.

Table 1: Galectin-4 serum levels and clinic-pathological features of patients with CC.

Cervical cancer	n	Gal-4 in serum (pg/ml)		p value
		Median	Range	
Histological type				
Squamous carcinoma	32	757.062	141.130-2518.000	0.0949
Adenocarcinoma	7	493.630	149.880-1388.000	
Differentiation				
Poor	6	539.285	316.430-1139.290	
Moderate	21	565.000	149.880-1636.430	ns
Well	3	1108.000	368.630-2518.000	
No reported	9			
Keratinizing				
No	14	630.030	141.130-1462.140	0.4677
Yes	10	757.062	381.130-1478.000	
Not reported	15			
FIGO staging				
I-II	17	563.630	141.130-1462.140	0.1997
III-IV	10	681.992	453.630-1356.429	
Not classifiable	12			
Tumor size (cm)				
≤5.1	8	613.155	198.630-1636.430	0.1325
>5.1	6	448.630	141.130-647.860	
No size	24			

p values were calculated using Mann–Whitney test with the exception of the differentiation grade which was calculated using Kruskal-Wallis test.

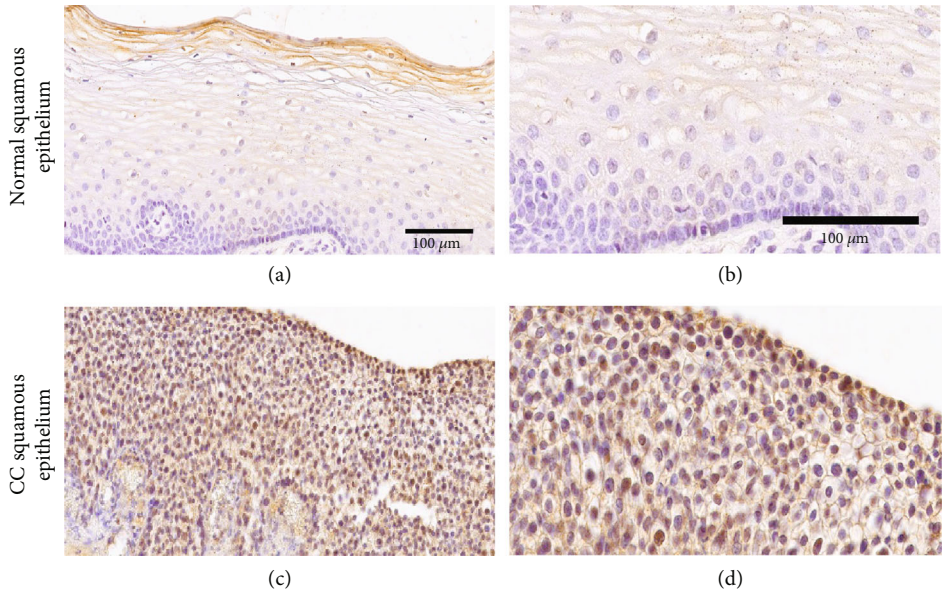

Figure 2: Galectin-4 immunodetection in cervical tissue samples from normal squamous epithelium and squamous CC. (a, b) correspond to normal squamous epithelium to 20x and 40x of magnification, respectively. The expression of galectin-4 was observed in the cytoplasm of the cells. (c, d) correspond to squamous CC to 20x and 40x of magnification, respectively. Galectin-4 was observed in both the cytoplasm and the nuclei of the tumor cells.

The serum levels of galectin-4 in CC patients were higher than those in normal serum. Serum levels of galectin-4 have been analysed in gastric adenocarcinoma [16], colon cancer, breast cancer [18, 23], and hepatocellular carcinoma (HCC) [6]. In all cancer types, the serum concentration of galectin-4 has been reported to be increased. In the case of colon cancer, the serum levels of galectin-4 were increased 11.1-fold overall and 25.3-fold in patients with metastasis with

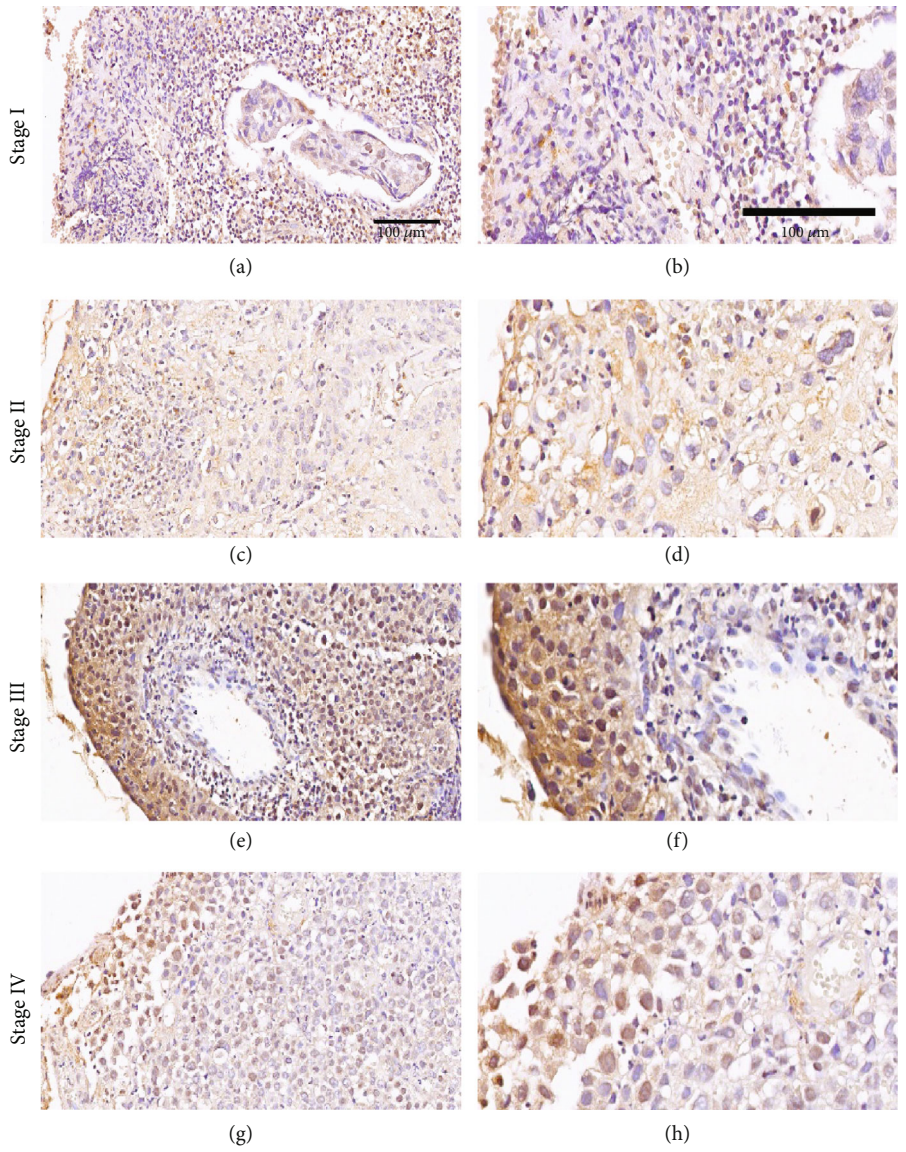

Figure 3: Galectin-4 expression in tumors from patients with different FIGO stages. Squamous cervical cancer tissue corresponds to (a, b) stage I, (c, d) stage II, (e, f) stage III, and (g, h) stage IV, to magnification 20x and 40x, respectively. Galectin-4 was observed in epithelial cells of tumors of patients with different FIGO stages, and its expression was observed in both the cytoplasm and nuclei. Galectin-4 was strongly expressed in some tumor cells with no relation to the FIGO stage.

respect to the levels in the healthy group; in breast cancer patients, an increase of 11-fold was detected [18]. With respect to hepatocellular cancer, galectin-4 was only increased in the serum of patients with HCC and infection with hepatitis B virus [6], and an association between higher serum levels and more aggressive characteristics of the cancer has also been reported. Our results are similar to those in other cancer types, in which serum levels of galectin-4 are increased in cancer patients compared with patients without disease. In addition, we observed a trend toward an increase in the serum concentration of galectin-4 in the patients with keratinizing tumors compared with nonkeratinizing tumors, as well as in FIGO stages III and IV with respect to FIGO stages I and II. In hepatocellular and colorectal carcinomas, an association has been reported between higher concentrations of serum galectin-4 and advanced clinical stage [6, 18]; additionally, in patients with hepatocarcinoma, higher serum concentrations of galectin-4 were associated with metastasis [23]. Our results suggest that galectin-4 could have a role in the invasion and metastasis of CC, but more serum samples from patients in stage IV must be analysed to confirm this association. The role of circulating galectin-4 was not characterized. *In vitro* studies suggest that circulating galectins could participate in recruiting tumor cells to the vascular endothelium. Circulating galectin-3 was shown to participate in metastasis in an *in vivo* model of nude mice. Galectin-3 binds to the oncofoetal Thomsen-Friedenreich antigen, which is a disaccharide (Galβ1,3GalNAc) present in the MUC1 transmembrane protein of tumor cells, and the interaction favours the exposure of other adhesion molecules, which facilitates the adhesion of tumor cells to the vascular endothelium [30].

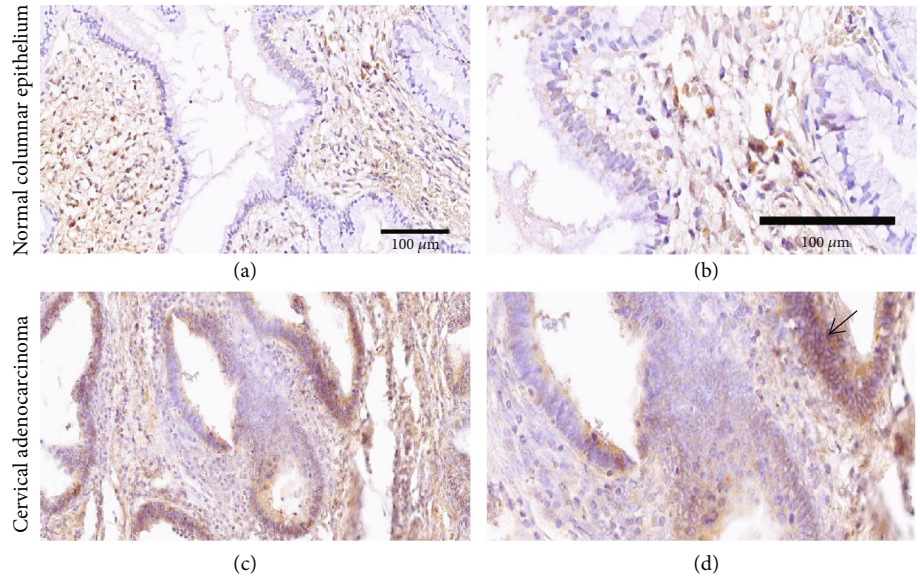

Figure 4: Galectin-4 expression in columnar cervical epithelium. (a, b) correspond to normal columnar epithelium and (c, d) to cervical adenocarcinoma. Galectin-4 was observed in columnar and stromal cells. In the columnar cells in nondiseased tissue, the expression was low, and its expression was increased in columnar cells in adenocarcinoma. In adenocarcinoma tissues, it was observed that the expression level of galectin-4 was increased in areas that had greater proliferation (arrow).

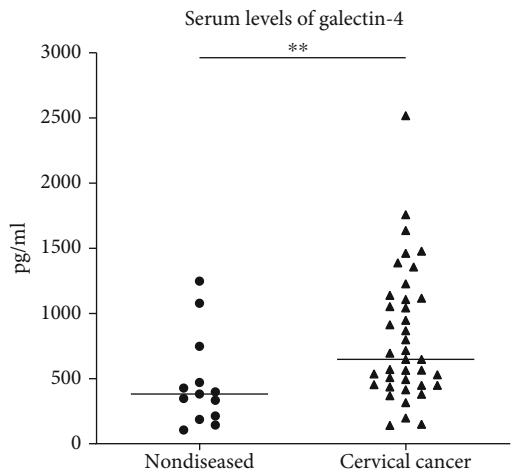

Figure 5: Tissue expression of galectin-4. Expression of galectin-4 in keratinizing (circle, $n = 16$) and nonkeratinizing (square, $n = 5$) CC tumors. The expression level of galectin-4 in keratinizing tumors was higher than that in nonkeratinizing tumors. The mean density of the expression level of galectin-4 was calculated with the Image-Pro software. Mann–Whitney test $^{*}p = 0.0318$.

It is important to mention that the deviation of serum galectin-4 values in CC patients was high; in some cases, the serum levels in patients with CC were similar to those in healthy women, and in other cases, the serum levels in patients with CC were twice the values in healthy women. In gastric adenocarcinoma patients, values up to 40-fold the values in healthy people have been reported in some cancer patients, while other patients have similar values to healthy people [16]. These differences could be associated with cancer progression and could be utilized for early identification of patients with a higher risk of cancer progression, and it has been proposed that circulating galectins could be therapeutic targets to reduce metastasis [16].

Galectin expression has also been reported to be altered in the tissues of different types of cancer. Galectin-1 [31, 32], galectin-3 [33, 34], and galectin-9 [35–37] are the most studied. The galectin-4 expression has been detected in different tissues and was first reported in the alimentary tract and liver [38]. During cell transformation, the expression of galectin-4 changes, and in some types of cancer, its expression has been related to stage or prognosis [6, 39].

Immunohistochemical staining showed that galectin-4 was preferentially expressed in the cytoplasm of normal epithelial cells, and its expression in SCC cells was observed in the cytoplasm and nuclei, showing changes in the localization associated with cancer transformation. With respect to the normal columnar cells, the faint expression of galectin-4 was detected in the cytoplasm. In adenocarcinoma, the expression was higher than that in normal columnar epithelium, and it was also observed primarily in nuclei. For some galectins, changes in the intracellular localization associated with cancer have been reported; galectin-7 was detected in nuclei in normal oesophageal epithelial tissue, and in oesophageal cell carcinoma, its expression was detected in the cytoplasm, nuclei, and membrane [12]. The preferential nuclear localization of galectin-3, over cytoplasmic localization, was proposed as a prognostic indicator of recurrence in lung carcinoma patients [11]. Galectin-1 induces the migration of mammary epithelial tumor cells, and this influence is exercised when the protein is located in the nucleus. It was also reported that the change in localization is the result of glycosylation, showing that α2,6 sialylation interferes with the interaction of galectin-1 with LacNAc glycans, allowing the translocation of galectin-1 to the nucleus [31]. During cervix

Table 2: Expression levels of galectina-4 in tissue and clinic-pathological features of patients with CC.

Cervical cancer	n	Expression levels of Gal-4 (lum) Median	Range	p value
Histological type				
Squamous carcinoma	23	72.216	4.129-151.793	
Adenocarcinoma	5	61.985	11.779-90.595	Squamous carcinoma vs. adenocarcinoma 0.3272
Adenosquamous carcinoma	1	22.053	NA	
Differentiation				
Poor	2	80.560	NA	Poor vs. moderate 0.6403
Moderate	21	71.028	4.129-151.793	
Well	1	90.575	NA	
No reported	5			
Keratinizing				
No	16	62.226	4.129-129.247	0.0318
Yes	5	102.896	55.008-110.984	
Not reported	8			
FIGO staging				
I-II	16	57.434	4.129-151.793	0.1868
III-IV	9	78.020	19.747-120.247	
Not classifiable	4			
Tumor size (cm^2)				
≤5.1	9	61.985	4.129-90.575	0.7197
>5.1	10	62.115	9.532-77.060	
No size	10			

[a]p values were calculated using Mann–Whitney test.

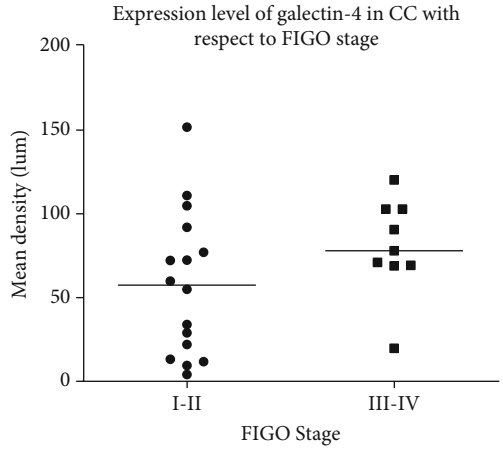

Figure 6: Tissue expression of galectin-4 with respect to FIGO stage. The expression of galectin-4 in tumors of patients with FIGO stages I/II (circle, $n = 16$) and III/IV (square, $n = 9$) is shown. The lowest galectin-4 levels were observed in FIGO stages I and II, and FIGO stages III and IV presented a trend of higher levels than FIGO stages I and II. The mean density of the expression level of galectin-4 was calculated with Image-Pro. The Mann–Whitney analysis did not show significant differences ($p = 0.1868$).

transformation, increased expression of sialic acid has been reported according to the transformation grade [40, 41]. Increased sialic acid in CC tissue can participate in the translocation of galectin-4 to the nucleus. The expression analysis of galectin-4 showed a significant difference between keratinizing and nonkeratinizing tumors, and the keratinizing tumors presented increased staining. The Cancer Genome Atlas Research Network reported a molecular characterization of CC tumors. The results showed three CC clusters: the keratin-high group, which presents a high expression of genes related to the keratin family; the keratin-low group, which presents low expression of keratin family genes; and the adenocarcinoma-rich group. These classifications show that it is possible to identify different subtypes of CC according to gene expression patterns [42]. In our results, we detected increased expression of galectin-4 in keratinizing tumors compared with nonkeratinizing tumors, suggesting that the galectin-4 gene could be part of the cluster of genes with high expression and play a role in this molecular subtype. To confirm this possibility, more studies are necessary. It has been reported that patients with the keratinizing type of SCC have advanced stages (III-IV) of disease; in addition, stage IV disease is also related with a worse survival rate, suggesting that keratinization status is an independent predictor of survival [43].

These results highlight the importance of considering molecular subtypes in the prognosis of CC and for designing targeted therapies.

The role of galectin-4 in CC tissue is still unknown, and the nuclear galectin-4 function in cancer progression must be investigated.

5. Conclusions

The higher serum levels of galectin-4 in the CC patients than in patients without disease and the trend of higher concentration in the patients with advanced clinical stage suggest that galectin-4, like other galectins, could participate in migration and metastasis.

Galectin-4 is expressed in cervical columnar and normal squamous epithelium and in stromal cells. In the normal tissue, the expression of galectin-4 was detected in the cytoplasm; in the samples from SCC and adenocarcinoma tumors, the expression was observed in nuclei, suggesting that the localization change could be related to cell transformation. The increased expression of galectin-4 in keratinizing tumors compared with nonkeratinizing tumors suggests that this gene could be part of the cluster of genes related to this CC subtype and could be related to worse prognosis.

Data Availability

The data analysed herein are included in the text.

Conflicts of Interest

The authors declare that they have no competing interests.

Acknowledgments

This work was supported by the Fondo de Investigación en Salud, IMSS (grant number FIS/IMSS/PROT/G17-2/1760) and the Consejo Nacional de Ciencia y Tecnología, Infraestructura (grant number 300379). ICR received a scholarship from the Mexican Institute of Social Security (98227566) and CONACYT (553529).

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