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Relevant Role of Di-Fucosylated Ley Antigen in the Outcome of Locally Advanced Cervical Squamous Cell Carcinoma Patients Treated with Cisplatin Regimen

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Abstract

Background: Several mechanisms are involved in the development of resistance to therapy in LACSCC. Studies have shown that CD44 and Lewis Y antigen (LeY) form a complex that is associated with chemoresistance, tumor invasion and metastasis. We assessed the role of CD44 and LeY in the outcome of LACSCC patients (pts) treated with different chemotherapy regimens.

Methods: A total of 126 LACSCC pts FIGO stages IIB-IVA were selected from GOCS databases: 74 pts included in three different prospective phase II trials in the neoadjuvant setting (vinorelbine, docetaxel, ifosfamide-vinorelbine-cisplatin) and 52 pts treated with standard radio-chemotherapy based in cisplatin (RCBC). Clinical data at baseline, disease free survival (DFS) and overall survival (OS) were recorded. Univariate and multivariate Cox models were employed.

Results: Median age was 45.6 years (range: 24.9 - 80.5). Sixty-three and 47 tumors were CD44+ and LeY+, respectively. Expansive growth tumors showed a higher grade (p=.0024), mitotic index (p=.0505), tumoral necrosis (p=.0191), LeY+ (p=.0034) and CD44+/LeY+ co-expression (p=.0334). CD44+ cells were present in 91.3% of those with local recurrence (p=.0317). Advanced stage was associated with LeY+ tumors (p=.0057). Pts treated with RCBC had worse DFS and OS when their tumors expressed LeY antigen (p=.0083 and p=.0137, respectively). Pre-treatment hemoglobin level, FIGO stage and tumor response remained the most significant prognostic factors in Cox regression.

Conclusions: In our cohort of LACSCC pts, the co-expression of CD44+/LeY+ was not associated with worse outcome. However, in the subgroup of pts receiving RCBC, LeY expression was correlated with shorter DFS and OS.

Keywords: Cervical cancer; CD44; Lewis Y antigen; Predictive factors

Introduction

Cervical cancer is one of the most frequent malignancies in women and a considerable cause of morbidity and mortality. It is the second most frequent type of cancer in women worldwide, only preceded by breast cancer [1]. Each year approximately 500,000 women around world are diagnosed with invasive cervical cancer and more than half of them die from their disease. Eighty percent of these deaths occur in developing countries. Therefore, it is essential for us to reach a deeper understanding of the biology of this disease in order to develop more effective therapies.

The development of resistance to chemo- or radio-therapy involves multiples mechanisms in locally advanced cervical squamous cell carcinoma (LACSCC). One of them could be the presence of a subpopulation of cells with regenerative abilities under cytotoxic stress. Such cancer stem or clonogenic cells tend to repopulate tumors during the course of chemo- or radiotherapy [2,3].

CD44 is a cell adhesion glycoprotein functioning as a transmembrane receptor for extracellular matrix component hyaluronan (HA). It participates in epithelial cell-stroma interactions that are important in tumour invasion and metastasis [4]. CD44 expression characterizes a subset of cancer cells with stem-cell-like properties and could be involved in cell adhesion-mediated drug resistance (CAM-DR) through interaction with its ligand HA [5,6]. It is known that CD44 is modified post-translationally by glycosylation, which has shown to influence CD44-mediated CAM-DR [7].

Recently, Gao et al. found that the di-fucosylated Lewis Y (LeY) antigen is part of the composition of CD44 and their increased expression is correlated with enhanced CD44-mediated cell adhesion and migration [8]. Also, increased expression of LeY antigen and CD44 were associated with elevated resistance to chemotherapeutic drugs such as platinum agents, taxanes, 5-fluorouracil, doxorubicin and mitomycin [9-12].

Although the effects of alternative splicing and post-translational glycosylation of CD44 on its interaction with HA have been studied in several tumor types, few data has described the effect of these alterations in LACSCC. The aim of this study is to evaluate the role of CD44 and LeY antigen in the outcome LACSCC patients.

Patients and methods

Patients: One hundred twenty six women with histologically proven diagnosis of squamous cell carcinoma for uterine cervix, treated at the

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GOCS group between January 1993 and December 2007 were included. Patients presented with International Federation of Gynecology and Obstetrics (FIGO) stages IIB to IVA.

Among the 126 patients, 74 participated in one of three different prospective phase II trials conducted by our group in the neoadjuvant setting: 23 received vinorelbine and 25 docetaxel (antimicrotubules agents), 26 ifosfamide plus vinorelbine plus cisplatin (mixed agents). The remaining 52 patients received cisplatin at standar doses concurrent with pelvic RT. Eligibility criteria, clinical staging, response criteria, treatment strategy, toxicity evaluation as well as scheme of doses, number of cycles of chemotherapy, criteria for surgery or radiotherapy were described in previous reports from our group [13-15].

Formalin-fixed paraffin-embedded tissues were obtained from 126 primary cervical carcinomas before treatment initiation. The original hematoxylins of all the cervical cancer cases were reevaluated to corroborate the original diagnosis and to classify the cases according to the original epithelial tissue. Tumoral samples with any of the following characteristics were excluded: excessive necrosis and bleeding, insufficient tumor material, poorly preserved tumor material, and those cases in which there were no corresponding paraffin blocks. Ten cases of benign exocervical and endocervical tissue were included as a control group to compare the inmunohistochemical reactivity of cervical cancer.

All procedures followed the World Medical Association Declaration of Helsinki and its further modifications. Informed consent was obtained from all patients. This study was approved by the Regional Ethical Committee of the province of Neuquen, Argentina (CAIBSH # 2520/11). The REMARK guidelines (Reporting recommendations for tumor MARKer prognostic studies) were followed [16].

Clinical and histological information for cervical cancer patients are summarized in Table 1.

Immunohistochemical analysis: The technique was performed following standard procedures. All specimens were fixed in formalin and embedded in paraffin. Sections were deparaffinized in xylene and hydrated in a graded ethanol series; dewaxed sections were placed in methanol with hydrogen peroxide (3%) for 15 minutes to block endogenous peroxidase activity. After three washes with PBS, sections were blocked for nonspecific binding with 50 ml of normal horse diluted 1:10 in 1% bovine serum albumin/PBS for 15 minutes and rinsed.

Prior to immunostaining with monoclonal antibodies (MAbs), tissues were treated with 10 mM sodium citrate buffer at 100°C for 5 minutes for antigenic retrieval. Afterwards, the sections were incubated with 50 µl of the mouse MAbs against standard CD44 (clone DF1485, 1:200; Santa Cruz) and Lewis Y (clone A70-C/C8, 1:200; Santa Cruz) at room temperature 60 minutes in a moist chamber. After three rinses with PBS, the sections were incubated with peroxidase-conjugated anti-mouse/rabbit Igs (HiDef DetectionTM HRP Polymer System, Cell Marque) for 60 minutes. After being washed, slides were counter-stained with hematoxylin, dehydrated in ethanol, washed with xylene and coverslipped with mounting media. Negative controls were performed by adding PBS instead of the primary antibody. The positive reaction against the antibodies in the study was represented by the presence of brown precipitation in the membrane or cytoplasm or in both cellular compartments.

Evaluation of staining: Specimens were examined with a light microscope by two independent observers (GG and AZ), who had no knowledge of the patient´s clinical data. The antibody staining patterns

		Cases (%)	5-years OS (%)	DFS	OS
				p-value*	p-value*
Age (years)	≤40	42 (33)	30.6	0.5153	0.1786
	>40	84 (67)	41.1		
Pre-treatment Hb (g/L) level	<115	40 (37)	19.3	0.0009	0.0003
	≥115	68 (63)	54.5		
FIGO stages	II	63 (50)	51.0	0.0008	0.0005
	III-IV	63 (50)	22.4		
Tumor size (cm)	≤4	42 (33)	30.6	0.4416	0.3682
	>4	84 (67)	41.1		
Mitotic index	<40	73 (58)	36.5	0.5695	0.6391
	≥40	53 (42)	37.5		
Necrosis	No	90 (71)	39.9	0.4622	0.2633
	Yes	36 (29)	28.7		
Tumor grade	1	22 (17)	39.4	0.5520	0.8496
	2	88 (70)	35.8		
	3	16 (13)	44.4		
Nuclear grade	1	13 (10)	20.5	0.3654	0.5579
	2	83 (66)	39.5		
	3	30 (24)	46.0		
Keratinizing	No	61 (48)	35.3	0.9347	0.6812
	Yes	65 (52)	37.8		
Viral cytopathic effect	No	110 (87)	35.9	0.8265	0.9361
	Yes	16 (13)	45.4		
Growth pattern	Infiltrative	84 (76)	29.6	0.0312	0.0763
	Expansive	27 (24)	42.0		
Treatment	CT→ RT	66 (52)	33.9	0.0130	0.0418
	CT→ RH	15 (12)	48.1		
	CT + RT	30 (24)	49.2		
	Others#	15 (12)	12.3		
Surgery	No	104 (82)	36.2	0.2115	0.2293
	Yes	22 (18)	42.6		
Radiotherapy	No	20 (16)	45.4	0.8544	0.9602
	Yes	106 (84)	36.1		
Chemotherapy	DTX	25 (20)	35.4	0.4450	0.6302
	VNB	23 (18)	38.6		
	CDDP-VNB-IFX	26 (21)	31.3		
	CDDP	52 (41)	41.0		
UICC	CR	21 (17)	88.2	0.0001	0.0001
	PR	26 (21)	60.0		
	SD	12 (9)	8.3		
	PD	32 (25)	3.4		
	Unknown	35 (28)	45.1		
CD44	Neg	63 (50)	37.1	0.9307	0.5956
	Pos	63 (50)	39.1		
LeY	Neg	79 (63)	34.4	0.8619	0.9483
	Pos	47 (37)	41.7		
Immunophenotype	$CD44^+/LeY^-$	44 (35)	42.2	0.3819	0.7787
	$CD44^-/LeY^+$	28 (22)	48.1	0.4866	0.3002
	$CD44^+/LeY^+$	19 (15)	31.1	0.2968	0.2644
	$CD44^-/LeY^-$	35 (28)	29.6	0.4576	0.7121

Table 1: Clinical features and survival in 126 patients with LASCC according to the investigated variables.

were scored in a semiquantitative manner. Low-power images of sections were scored based on the staining intensities: negative (-), low (+), moderate (++) and strong (+++), using adjacent non-malignant cells for reference. Normal tonsils were used as a positive control for CD44, and colon cancer tissue was used as positive control for LeY, according to the manufacturer's recommendations. Negative controls for immunostaining were prepared through serial sections of selected tissue samples in the absence of the primary antibody.

Subsequently, a total of 5 high-power fields in series were selected from each slide for scoring of individual cells. The mean percentages of positive stained cells were calculated for each field. Percentage of positive cells was graded as follows: 0, no positive cells; 0.1, positive in less than 9% of cells; 0.5, positive in 10-49%; 1, positive in more than 50% of cells. The pattern of reaction was classified as linear (membrane), cytoplasmic and mixed (cytoplasmic with plasma membrane staining). Apical and non-apical staining, adjacent normal tissue, and focal or diffuse staining were all evaluated.

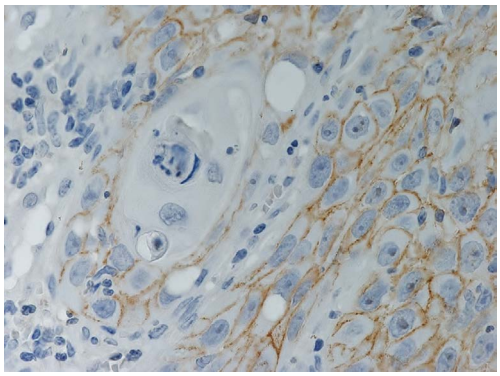
Different cut-off according to previous reports, taking account the intensity and percentage of CD44 and LeY, were tested. No meaningful results were found when considered as positive or negative the immunostaining of both antigens. For prognostic investigation and survival analysis, each individual immunophenotype pattern was evaluated: CD44+ (CD44-positive cells), LeY+ (LeY-positive cells), CD44+/LeY+, CD44+/LeY-, CD44-/LeY+ or CD44-/LeY-.

Statistical analysis: The associations of CD44 and LeY expression with the variables studied were tested with non-parametric tests. For categorical variables, the Wilcoxon rank-sum test or Kruskal-Wallis test, including a Wilcoxon-type test for trend across ordered groups were used.

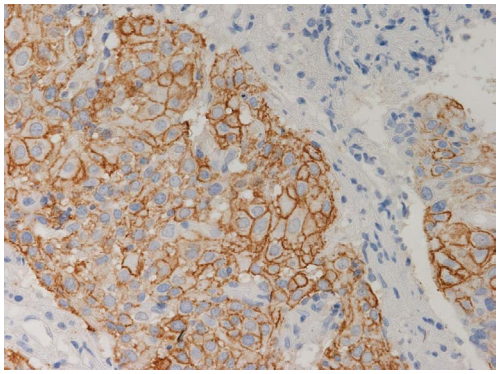
Disease free survival (DFS) was estimated from the date of diagnosis until the date of first tumor recurrence. Overall survival (OS) was calculated from the date of diagnosis until the date of death or last follow-up. The evaluation of treatment response was performed using the International Union for International Cancer Control (U.I.C.C.), standard method used in solid tumors until 2000.

DFS and OS univariate associations with CD44 and LeY expression were evaluated employing the Kaplan Meier estimator with log rank test. A multivariable Cox regression model addressed OS and DFS associations among CD44+, LeY+, CD44+/LeY+, CD44+/LeY-, CD44-/LeY+, CD44-/LeY-, age (≤40 vs. >40), pre-treatment hemoglobin level (<115 g/L vs. ≥115 g/L), FIGO stage (early II vs. advanced III-IV), tumor size (≤4 cm vs. >4 cm), mitotic index (<40 vs. ≥40), histological grade (low 1-2 vs. high 3), nuclear grade (low 1-2 vs. high 3), keratinizing (yes vs. no), viral cytopathic effect (yes vs. no), tumoral necrosis (yes vs. no), and growth pattern (infiltrative vs. expansive).

All variables where normalized and significance was set at $p<0.05$. All calculations were done employing the Statistix v8. software package.



(A)



(B)

Figure 1: An invasive squamous cervical carcinoma sample showing membranous staining of CD44 (A) and LeY antigen (B) Original magnification x 200.

Results

Patient characteristics associated with prognosis

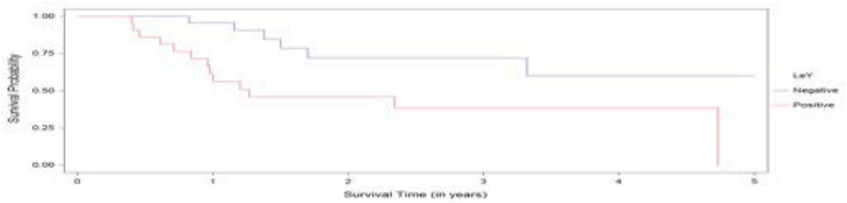
One hundred and twenty-six patients with LACSCC with a median age of 45.6 years (range: 24.9 - 80.5 years) were included. Sixty-three patients (50%) had FIGO stage IIB whereas the reminder had advanced stage (52 patients were FIGO stage IIIB, 7 IVA and 4 IVB). Fifty-six patients (44.4%) showed local recurrence and 71 patients (56.3%) died from their disease. Univariate analysis showed that hemoglobin level lower than 115 mg/dl, advanced stages, expansive tumoral pattern, treatment and poor response by UICC criteria were related with worse DFS and OS. The other clinicopathological characteristics are summarized in (Table 1).

Relationship of CD44 and LeY expression with resistance to therapy

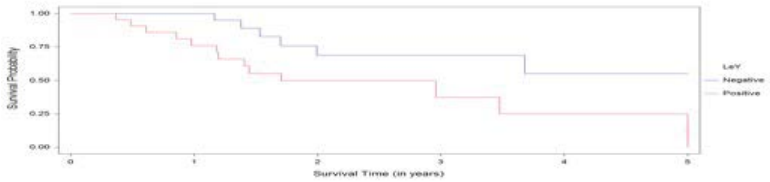
Membrane immunoexpression was seen in all CD44+ squamous cervical cancer, predominantly weak intensity, and in a focal area of tumor (Figure 1). Only 23/63 (36.5%) tumors expressed CD44 in more than 10% of tumoral tissue. In the underlying stroma, the endothelial CD44 positive cells were taken account as internal positive control. LeY antigen expression was observed in 47/126 (37.3%) of tumoral tissues. Twenty-nine (61.7%) of them showed membrane (Figure 1), 13 (27.7%) cytoplasmatic, and 5 (10.6%) mixed (membrane and cytoplasmatic) immunostaining. In 36 (76.3%) tumors the intensity of LeY was weak and 40 (85.1%) in a focal area (Figure 2). The positive staining of polymorphonuclear leukocytes was taken in account as internal positive control. The immunophenotypes tumors analyzed according to clinicopathologic features are demonstrated in Table 2.

The predominant immunophenotypes were CD44+ (50%), LeY+ (37.3%), and CD44+/LeY- (34.9%). CD44+ cells were present in 13/16 (81.3%) of tumors with viral cytopathic effect and in 21/23 (91.3%) of those with local recurrence. The patients with advanced stages expressed higher LeY+ (66% vs. 34%, p=.0057) and CD44+/LeY+ (68.4% vs. 31.6%, p=.0814) than those with early stages.

Expansive growth tumors presented significantly higher grade (p=.0024), mitotic index (p=.0505), tumoral necrosis (p=.0191), LeY+ (p=.0034) and CD44+/LeY+ co-expression (p=.0334) (data not showed).



(A)



(B)

Figure 2: DFS (A) and OS (B) curves according to LeY expression in the subgroup of patients receiving radiochemotherapy based in cisplatin (Kaplan-Meier).

		CD44$^+$	LeY$^+$	CD44$^+$/LeY$^-$	CD44$^-$/LeY$^+$	CD44$^+$/LeY$^+$	CD44$^-$/LeY$^-$
		n=63 (%)	n=47 (%)	n=44 (%)	n=28 (%)	n=19 (%)	n=35 (%)
Age (years)	≤40	22 (35)	16 (34)	14 (32)	8 (29)	8 (42)	12 (34)
	>40	41 (65)	31 (66)	30 (68)	20 (71)	11 (58)	23 (66)
	χ^2, p-value	0.7055	0.8964	0.7916	0.5445	0.3788	0.8882
Pre-treatment Hb (g/L) level	<115	21 (37)	14 (33)	14 (38)	7 (29)	7 (37)	12 (43)
	≥115	35 (63)	29 (67)	23 (62)	17 (71)	12 (63)	16 (57)
	χ^2, p-value	0.9177	0.4331	0.9010	0.3653	0.9845	0.4587
FIGO stages	II	30 (48)	16 (34)	24 (54)	10 (36)	6 (32)	23 (66)
	III-IV	33 (52)	31 (66)	20 (46)	18 (64)	13 (68)	12 (34)
	χ^2, p-value	0.5930	0.0057	0.4548	0.0865	0.0814	0.0287
Tumor size (cm)	≤4	22 (35)	16 (34)	14 (32)	8 (29)	8 (42)	12 (34)
	>4	41 (65)	31 (66)	30 (68)	20 (71)	11 (58)	23 (66)
	χ^2, p-value	0.7055	0.8964	0.7916	0.5445	0.3788	0.8882
Mitotic index	<40	37 (59)	25 (53)	26 (59)	14 (50)	11 (58)	22 (63)
	≥40	26 (41)	22 (47)	18 (41)	14 (50)	8 (42)	13 (37)
	χ^2, p-value	0.8568	0.4053	0.8475	0.3347	0.9968	0.4878
Necrosis	No	44 (70)	36 (77)	30 (68)	22 (79)	14 (74)	24 (69)
	Yes	19 (30)	11 (23)	14 (32)	6 (21)	5 (26)	11 (31)
	χ^2, p-value	0.6933	0.3220	0.5546	0.3428	0.8133	0.6597
Tumor grade	1	11 (17)	9 (19)	7 (16)	5 (18)	4 (21)	6 (17)
	2	41 (66)	34 (72)	28 (64)	21 (75)	13 (68)	26 (74)
	3	11 (17)	7 (9)	9 (21)	2 (7)	2 (11)	3 (9)
	χ^2, p-value	0.2646	0.5421	0.1597	0.6019	0.8803	0.6737
Nuclear grade	1	6 (9)	7 (15)	4 (9)	5 (18)	2 (11)	2 (6)
	2	39 (62)	30 (64)	27 (61)	18 (64)	12 (63)	26 (74)
	3	18 (29)	10 (21)	13 (30)	5 (18)	5 (26)	7 (20)
	χ^2, p-value	0.4543	0.4125	0.5375	0.2818	0.9584	0.4043
Keratinizing	No	26 (41)	22 (47)	19 (43)	15 (54)	7 (37)	20 (57)
	Yes	37 (59)	25 (53)	25 (57)	13 (46)	12 (63)	15 (43)
	χ^2, p-value	0.1086	0.7811	0.3894	0.5357	0.2734	0.2239
Viral cytopathic effect	No	50 (79)	42 (89)	35 (79)	27 (96)	15 (79)	33 (94)
	Yes	13 (21)	5 (11)	9 (21)	1 (4)	4 (21)	2 (6)
	χ^2, p-value	0.0075	0.5922	0.0554	NA	0.2353	0.1442

Growth pattern	Infiltrative	14 (26)	4 (9)	13 (36)	3 (12)	1 (5)	10 (31)
	Expansive	41 (74)	39 (91)	23 (64)	21 (88)	18 (95)	22 (69)
	χ^2,p-value	0.7833	0.0034	0.0449	0.1272	NA	0.2791
Recurrence of disease	No	40 (63)	24 (51)	29 (66)	13 (46)	11 (58)	17 (49)
	Yes	23 (37)	23 (48)	15 (34)	15 (54)	8 (42)	18 (51)
	χ^2,p-value	0.0730	0.4338	0.0867	0.2704	0.8238	0.3279
Type of recurrence	Local	21 (91)	18 (78)	14 (93)	11 (73)	7 (87)	11 (69)
	Distant	2 (9)	5 (22)	1 (7)	4 (27)	1 (13)	5 (31)
	χ^2,p-value	0.0317	0.8272	NA	0.7113	NA	0.0559

Table 2: Relationship between immunomarkers and clinicopathological features of LACSCC patients.

	Neoadjuvant therapy						**Radiochemotherapy**		
	Antimicrotubules agents			**Mixed agents**			**Cisplatin**		
	cases	DFS	OS	cases	DFS	OS	cases	DFS	OS
	48	p-value*	p-value*	26	p-value*	p-value*	52	p-value*	p-value*
CD44 $^+$	20	0.4964	0.3674	13	0.9185	0.7509	30	0.4633	0.6385
LeY$^+$	17	0.3674	0.1712	6	0.7021	0.7111	24	0.0083	0.0137
CD44$^+$/Ley$^+$	3	0.2241	0.3970	4	0.5987	0.6212	12	0.1578	0.1670
CD44$^+$/Ley$^-$	17	0.9315	0.6168	9	0.9143	0.4787	18	0.0598	0.0870
CD44$^-$/Ley$^+$	14	0.0635	0.0586	2	0.9143	0.9334	12	0.0868	0.1245
CD44$^-$/Ley$^-$	14	0.2635	0.3610	11	0.8717	0.7142	10	0.3622	0.2923

Table 3: Survival analysis of tumor phenotypes according to chemotherapeutic agents used.

Mean follow-up was 3.4 years (range 0.1 to 18.4 years). The median overall survival time was 1.6 years. The 3-year DFS rate for all 126 patients was 40.8%, and the 5-year OS was 37.1%. There was no difference in DFS or OS according to CD44 or LeY status (Table 2). However, patients treated with radio-chemotherapy based in cisplatin had worse DFS and OS when their tumors expressed LeY antigen (p=.0083 and p=.0137, respectively) (Table 3, Figure 2). Also, the absence of LeY in cells CD44+ showed a trend to a better DFS than those with presence of LeY in CD44- tumors although these associations were not statistically significant.

Disease progression was registered in 56/126 (44.4%) LACSCC patients of which 43/56 (76.8%) presented local recurrence and 13/56 (23.2%) distant disease. The CD44 expression, in pretreated samples, was significantly higher in those tumors that progressed locally than those that developed distant metastasis (91.3% vs. 8.7%, p=.0317).

Multivariate analysis

The Cox proportional hazard regression model confirmed the variables that had significant association in the univariate analysis (Table 4).

Discussion

Few advances have been achieved in cervical cancer therapy over the past 20 years. In the late 1990s, investigators proved that the addition of concurrent cisplatin based chemotherapy to standard radiation therapy protocols could reduce recurrence and disease-related death rates by as much as 50% [17]. Other trials suggested that there might be a benefit for neoadjuvant chemotherapy using different therapeutic agents [18]. A decade later, an added survival advantage was reported when concurrent and adjuvant gemcitabine were combined with weekly cisplatin [19]. Today, combination of radiation therapy and chemotherapy is standard for patients with locally advanced cervical cancer, with an established improvement in OS. Although significant reduction in the relative risk of cancer death has been achieved, the absolute gains are relatively small for patients with early tumors, many of whom would have been cured with radiation alone, and recurrence rates are still high for patients who have very large or advanced-stage tumors. As with other tumor locations (i.e. breast, colon or lung cancer), in-depth knowledge of the biological behavior of squamous cervical cancer, and its response to different chemotherapeutic drugs, could help us select the best treatment for individual patients.

In order to evaluate chemoresistance to diverse chemotherapeutic agents used in cervical cancer, we selected 126 patients with LACSCC from GOCS database. The objective of this study was to determinate immunohistochemically in pretreated samples, whether CD44, LeY or their co-expression would contribute to the resistance to diverse agents used in the treatment of LACSCC patients.

In our series, we found a negative correlation between LeY antigen expression and the outcome of LACSCC patients who received radiochemotherapy based in cisplatin. Multiple mechanisms are involved in the resistance of cancer cells to cisplatin, including the expression of multidrug resistance-associated protein (MRP), enhanced DNA repair activity and alterations in signal transduction pathways, among others [20]. Studies have reported that protein glycosylation could play an important role in drug resistance. For example, in head and neck squamous cell carcinoma the aberrant glycosylation of α5β1-integrin is involved in the resistance to cisplatin as well as tunicamycin [21]; in ovarian cancer the overexpression of LeY antigen might be related with carboplatin/paclitaxel-resistant tumors [22]. Also, in colon carcinoma cells the α1, 2-fucosyltransferase and histoblood group antigen H type 2 are involved in resistance to 5-fluorouracil [23]. Recently, Gao et al. showed that LeY antigen is part of the composition of

Features	Categories	HR	SE	Z	P-value	Lower 95%C.I.	Upper 95%C.I.
Pre-treatment Hb (g/L) level	<115 vs. ≥115	0.31785	0.10697	-3.41	0.001	0.16435	0.61473
FIGO stages	II vs. III	110,238	0.40334	0.27	0.790	0.53813	225,826
	II vs. IV	603,718	341,089	3.18	0.001	199,490	182,703
UICC	CR vs. PR	838,572	881,930	2.02	0.043	106,740	658,799
	CR vs. SD	546,160	583,035	3.75	0.001	673,982	442,580
	CR vs. PD	399,418	422,678	3.48	0.001	501,944	317,833

Table 4: Multivariate analysis.

CD44 and increased levels of LeY antigen are associated with increased CD44-mediated epithelial ovarian cancer cells adhesion and migration as well as increased resistance to carboplatin and paclitaxel [8,24]. In our squamous cervical cancer study, we could not confirm this result. We did not find a clear association between CD44+/LeY+ tumors and the resistance to different chemotherapies used neoadjuvantly or in combination with radiotherapy. However, CD44+/LeY- and CD44-/LeY+ phenotypes showed an interesting trend to better and worse response to radiochemotherapy based in cisplatin, respectively. We also found a trend to worse response to docetaxel and vinorelbine (antimicrotubules agents) in CD44-/LeY+ tumors. Taken together, these findings suggest that the presence of di-fucosylated LeY antigen in squamous cervical cancer plays a negative role in chemoresistance, regardless of whether CD44 is present.

Studies have shown that glycosylated proteins are related to some malignant cell behaviors, including adhesion, recognition, and signal transduction; and that increased LeY antigens promote the invasion and migration of tumor cells [25]. Cervical tumors that extended to pelvic wall or compromised the vagina, bladder or rectum, or extended beyond true pelvis (FIGO stages III-IV) were associated to a significant increase of LeY expression in our study. Overexpression of LeY antigen could be responsible for an up-regulation of matrix metalloproteinases (MMPs), which lead to breakdown the extracellular matrix (ECM), and a down-regulation of specific tissue inhibitors of metalloproteinases (TIMPs) promoting the process of invasion although the specific mechanism still need to be further studied [26].

Several studies have analyzed the differences in CD44 expression in tumor areas and its correlation with prognosis of squamous tumors. Ostwald et al. observed major differences in the intensity of CD44 expression depending on the area of the tumor studied, with a high intensity of expression by cells of the external peripheral area and a low or absent expression in internal areas [27]. The authors concluded that CD44 can be a valid proliferation marker and attributed its higher expression at the periphery to the proliferative activity of malignant cells in these areas, which could indicate a tendency to expansive growth of the tumor. One reason for this growth pattern could be given by the increased expression of di-fucosylated antigen in the adhesion molecule CD44 [28]. Following this hypothesis, we observed a significant high expression of LeY antigen, and a predominant phenotype CD44+/LeY+, in tumors with expansive growth pattern, which showed also significantly higher mitotic index and necrosis than those with infiltrative pattern. Recently, Hotta et al. observed in squamous head and neck tumors an increased expression of LeY in superficial areas and decreased or absent expression in invasive regions which was correlated with the prognosis of patients [29]. However, others studies have demonstrated the opposite [30].

A number of clinical and morphologic factors have been well established to predict local recurrence in cervical cancers after radiochemotherapy, such as tumor size, histology, and regional spread. In addition, non-molecular (i.e. tumor hypoxia, interstitial tumor pressure, vascular density, anemia) and molecular (i.e. single-genes such as CAXII, ERCC1 and EGFR or HPV) biomarkers seem to be associated with local disease recurrence. In this context, we observed a positive correlation between higher expression of CD44 in the pretreated samples of LACSCC patients and the development of local recurrence. High CD44 expression was associated with local recurrence in many others tumors such as HER2-positive breast cancer [31], hepatocellular carcinoma [32], gastric cáncer [33], rectal cáncer [34] and larynx cancer [35]. A potential explanation for this phenomenon may include the fact that: 1) CD44 acts as co-receptor for ErbB family which can lead to activation of the *PI3K/AKT* pathway, a pathway known to promote survival after cytotoxic damage, including after radiotherapy suggesting a possible link between CD44 expression and intrinsic radiosensitivity; 2) CD44 could be links with hypoxia or repopulating ability, both known to influence radiotherapy outcome; and 3) CD44 expression could reflect the number of stem or cancer initiating cells needed to be killed independent of whether the putative stem cells are more or less radioresistant than bulk tumor cells.

The majority, 75-95%, of cervical tumors are positive for human papillomavirus (HPV). Interestingly, among populations with the rare case of cervical cancer that do not have detectable HPV, prognosis is poor [36]. Worse prognosis in HPV-negative tumors has also been reported in squamous oropharynx cancer, in which 30-40% of cases do not have detectable HPV. Five-year survival rates are 45-50% for patients with HPV-negative, as compared with 75-80% for those with HPV-positive tumors. According to these results, we found higher 5-years survival in LACSCC patients whose tumors present viral cytopathic effect than those without this characteristic, although the difference was not statically significant. The reason for the survival differences according to HPV status are not kwon, but HPV status correlates with multiple molecular abnormalities, including chromosomal changes and p53 mutation status [37]. Among of tumors with viral cytopathic effect found in our series, we observed a predominant expression of CD44 and the phenotype CD44+/LeY-. Recently, Näsman et al. observed in 290 oropharyngeal squamous cell carcinoma patients a poor DFS when the tumors had HPV+ and medium/strong CD44 intensity staining [38]. Also, Hufbauer et al. showed in a cutaneous keratinocyte line PM1 that the expose to HVP lead to a shift of the total population from low toward higher CD44 cell surface expression and, in turn, pointed to a reduction in the number of terminally differentiated cells [39]. The significant increase found in the number of CD44 cells suggests that HPV enlarge this population of proliferative stem cell-like cells. The authors concluded that the expression of HPV inhibits the entry of keratinocytes into differentiation and that the maintenance of basal cells in an undifferentiated state may increase the pool of cells available for the accumulation of damage that can persist and lead to the generation of stem cells with malignant properties. The genetic mutation resulting from high-risk HPV infection can lead the stem cells to undergo uncontrolled proliferation, invasion and metastasis.

In the univariate analysis, low pre-treatment hemoglobin level, expansive growth pattern, advanced FIGO stage, treatment and poor response to therapy were associated with shorter DFS and OS. Patients treated with radiochemotherapy based in cisplatin had worse DFS and OS when their tumors expressed LeY antigen. Pre-treatment hemoglobin level, FIGO stage and tumor response remained the most significant prognostic factors in Cox regression. In our series, we did not find any associations between CD44+/LeY+ tumors and the outcome. In line with our results, Lindstrom et al. assessed different biomarkers in 128 squamous cervical cancers and concluded that CD44 was not a valid prognostic marker [40].

In summary, in our cohort of LACSCC patients, the immunophenotype CD44+/LeY+ was not associated with worse outcome. However, in the subgroup of patients receiving radiochemotherapy based in cisplatin, LeY expression was correlated with shorter DFS and OS.

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