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SOX9 Immunosuppression in Primary Colorectal Cancer Tumors with Lymph Node Metastasis

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Abstract: Introduction and aims: Colorectal cancer is the most frequent malignant tumor of the digestive system. Its pathogeny is complex and involves the APC/β-catenin sequence. Lymph node metastases are a significant indicator for determining treatment and are a prognostic factor. SOX9 overexpression is related to oncogenic qualities and the capacity for metastasis. Our aim was to analyze SOX9 immunoexpression in primary colorectal cancer and lymph node metastasis status. Material and methods: Seventy-nine available cases were divided into the group with lymph node metastasis (n = 38) and the group without lymph node metastasis (n = 41), evaluating their SOX9 expression. The IBM SPSS version 27 program in Spanish was utilized to carry out the statistical analysis, obtaining measures of central tendency, the kappa index, standard deviation, Wilcoxon Mann-Whitney nonparametric measurements, Spearman's correlation coefficient, and chi-square test and Student's t test values. SOX9 immunoexpression was evaluated through the mean-based H-score, with high immunoexpression as a score ≥ 145 and low immunoexpression as a score ≤ 144. Results: A p = 0.73 was obtained that was not statistically significant, regarding the relation of SOX9 expression in primary colorectal cancer to lymph node metastasis. Conclusions: The absence or presence of lymph node metastasis was independent from SOX9 immunoexpression in primary colorectal cancer. However, due to the limited size of the population analyzed, further research is needed.

Key words: colorectal cancer; SOX9; immunoexpression; metastasis; lymph nodes

1. Introduction and Objectives

Colorectal carcinoma is the most frequent neoplasm of the digestive system and is defined as a malignant neoplasm of epithelial origin found in the large intestine [1]. Regarding its epidemiology, the Globocan 2020 [2] reports colorectal carcinoma in third place in frequency; the first place is occupied by breast carcinoma and the second by pulmonary carcinoma.

This neoplasm is divided according to glandular formation into well-differentiated (G1), moderately differentiated (G2), poorly differentiated (G3) and undifferentiated (G4) tumors [3]. For the staging of this neoplasm, histologic grading and the TNM system of the American Joint Committee on Cancer (8th edition) are used [4].

The pathogenesis of colorectal carcinoma is complex and presents diverse genetic and epigenetic mutations. Alteration in the APC/β-catenin sequence is part of the chromosome instability pathway, which is responsible for 65-70% of spontaneous colorectal carcinomas, and APC mutation the earliest alteration in colorectal carcinogenesis [5].

The molecular biology of colorectal cancer metastasis specifically refers to a continuous process. Primary tumors are described as composed of multiple different genetic subpopulations [6, 7]. Kwak et al. proposed the creation of new tissue pathological parameters that can evaluate the stromal microenvironment and predict metastasis using artificial intelligence [8].

Prognostic factors involved in this carcinoma include histologic type, tumor stage, presence of lymphovascular invasion, presence of tumor budding, host response, degree of response to treatment, status of surgical margins and, at the molecular level, the presence of microsatellite instability and genetic alterations including APC, TP53, SMDA4, PIK3CA, POLE, BRAF and RAS [1, 4, 9] of the carcinoma developmental pathway.

1.1 SOX9 in colorectal carcinoma

The sex determining region Y box-9 protein (SOX9) is a member of the SOX family of transcription factors. Regarding its involvement in the gastrointestinal tract, it has been shown in in vitro studies to participate in the differentiation of Paneth cells of the intestinal mucosa [10].

SOX9 has several implications in colorectal carcinoma, both as a biomarker in the early diagnosis of colorectal carcinoma and as a prognostic factor. It has been found that SOX9 blocks cell differentiation in the Wnt pathway by activating a stem cell-like program driven by specific enhancers [11]; likewise, the relationship of increased tumor budding in tumor stages Ⅲ and Ⅳ, high histological grade, with strong SOX9 expression has been observed [12]. Contreras Loera, in 2022, conducted an investigation in our institution on the association of SOX9 immunoexpression with the degree of differentiation of colorectal carcinoma [13], without obtaining statistical significance.

SOX9 associates with other proteins and genes that collaborate in its tumorigenic capacity. Among them is FARSA-AS1, a long non-coding RNA (lnc RNA) gene that is activated by SOX9, which is associated with cell growth and metastasis of colorectal carcinoma [14]. The MALAT1 lncRNA/miR-145/SOX9 axis was reported to mediate cell proliferation, migration and tumor invasion [15]. SOX9-mediated transcriptional activation of FOXK2 also participates in carcinogenesis by promoting tumor activity [16]; moreover, SOX9 is involved in predicting chemotherapy resistance through its interaction with DNMT1 [17]. Claudin 7 is a protein involved in cell adhesion and its low expression was demonstrated by cell culture, offering an enhancement in the oncogenic properties of SOX9 [18]. RAC1 is a transcription factor that can promote activation of the phospho-pentose pathway of colorectal carcinoma through SOX9/HK2/G6PD mediation [19]; ZRANB1 (tumor necrosis factor binding protein domain) is a deubiquitinase that controls cell growth and metastasis, which in turn increases SOX9 stability by decelerating its ubiquitination [20].

Analogously with the development of new therapeutic pathways, the authors have done studies on the administration of rapamycin (mTOR inhibitor) in those tumors that showed a high expression of the SOX9 biomarker [21]; likewise, it was demonstrated that high levels of SOX9 were associated with a lower response to 5-fluorouracil management by increasing vascular invasion and evidencing a therapeutic resistance to radiation [22, 23].

Based on the above, the main objective of this study was to evaluate the relationship of SOX9 immunoexpression in primary colorectal carcinoma and lymph node metastasis status.

2. Material and Methods

2.1 Study design and universe

An analytic, observational, cross-sectional study (according to STROBE) was conducted, in which paraffin blocks with a histopathologic diagnosis of colorectal cancer were used. The blocks were archived at the Department of Pathologic Anatomy of the secondary care institution, Hospital Central "Dr. Ignacio Morones Prieto", in San Luis Potosí, within the time frame of August 1, 2013, to August 31, 2022, providing a total of 79 cases.

Inclusion criteria: all cases with histopathological diagnosis of colorectal carcinoma with lymph node dissection, obtained by intestinal segment resection, were included.

Exclusion criteria: cases with another histopathological diagnosis, biopsy specimens and cases without lymph node dissection.

Elimination criteria: cases in which the paraffin blocks did not have the conditions to perform the immunohistochemistry study and those that did not have enough material for it were eliminated.

The sample size was small (n = 79), single-center and retrospective. This was due to two important reasons: the first was the COVID-19 pandemic, which paralyzed the care of many patients and their clinical and surgical treatment, leading to a low number of surgical specimens received in the pathology department; the other reason was the lack of a more appropriate space for the archiving of paraffin blocks that would allow the older blocks to be preserved in better quality and, finally, the removal of material at the request of the patient who owned the material.

2.2 SOX9 measured by the H-score

The nuclear immunoexpression of SOX9 in tumor cells was assessed in the areas of colorectal carcinoma corresponding to budding tumor, which were selected by consensus between the two reviewing physicians. A good concordance was obtained by kappa test with a score of 0.061.

To obtain the H-score, the nuclear immunolabeling of SOX9 was evaluated by multiplying the percentage of tumor cell reaction intensity by a value ranging from 0 to 3, and continued with the summation of these values to obtain the result; this result can range from 0 to 300 (% cells with null intensity × 0 + % cells with mild intensity × 1 + % cells with moderate intensity × 2 + % cells with strong intensity × 3 = 0 - 300) [24].

The cut-off point was established by obtaining the mean of the total H-score of the 79 cases [13, 17]. With this, an H-score score ≥ 145 was determined as high immunoexpression and a value ≤ 144 was determined as low immunoexpression.

2.3 Pilot test

Without knowing the characteristics of the population, a pilot study was conducted on paraffin blocks using the statistical program IBM SPSS Statistics 27, with the calculation characteristic of the population in 24 cases [25]. From this, a total of 12 cases with presence of lymph node metastasis and 12 cases without metastasis were obtained [25], in order to know the values of central tendency and the behavior of the population. Subsequently, based on the results obtained, the comparison of two proportions formula was used to calculate the sample [26].

We evaluated demographic aspects, pathologic characteristics of the tumor such as location, most frequent histologic type and grade, tumor size, lymphovascular and perineural invasion, pTNM classification, surgical edges and clinical characteristics such as previous treatment, overweight, sedentary lifestyle, smoking, alcoholism, diabetes mellitus and arterial hypertension.

2.4 Immunohistochemistry

For the immunohistochemistry study in paraffin blocks, the SOX9 antibody clone EP317 from BioSB was used, with a dilution of 1:200. The control tissue was colon and the localization of the immunoexpression evaluated was nuclear. The

procedure was performed following the antibody instructions, as detailed below.

Three slices measuring 3-4 microns were placed on Hydrophilic Plus Slides (BioSB) of the problem case and the respective tissue control. The slides were dewaxing overnight in an oven at 60°C. They were removed from the oven to cool. Next, the slides were placed in xylene for 10 min, passed through absolute alcohol at 96◦ and 80◦, and transferred to distilled water. They were then placed in a decloaking buffer solution (Immuno/DNA Retriever with EDTA, BioSB) and the antigen retrieval procedure was performed in an automated pressure cooker (Decloaking chamber, Biocare). The slides were cooled for 15-20 min, then rinsed for 5 min with distilled water and placed in hydrogen peroxide (3%) for 15 min. Once again, they were rinsed with distilled water for 5 min. They were placed in TBS with Tween and the slices were covered with the SOX9 antibody (100µl). They were placed in a humidified chamber and incubated for 30-40 min. The SOX9 antibody was diluted with TBSt, following the antibody dilution indications of 1:200, and rinsed with abundant TBSt. The slides were covered with biotinylated secondary antibody (biotin link) for 10 min and again rinsed with abundant TBSt. They were then covered with polymers (streptavidin-HRP) for 10 min and rinsed with abundant TBSt. Peroxidase activity was revealed under control microscopy, utilizing diaminobenzidine. The reaction was stopped by placing the slices in distilled water. Counterstaining was carried out with Harris hematoxylin for 30 s. Lastly, the slides were rinsed with running water, then in distilled water, and finally dehydrated until xylene to be covered with Entellan®.

2.5 Statistical analysis

The Spanish version of the IBM SPSS Statistics 27 program was used for the statistical analysis. Measures of central tendency were performed for the quantitative variables of the study [27]; the mean was calculated for the age variable and the mode was calculated for the frequency of sex, histologic type and location. Variability was calculated using the standard deviation; in addition, the kappa formula was applied to measure the concordance of the results of the 2 physicians who evaluated the H-score slides. For population characteristics, the chi-square test was used to evaluate pretreatment and sedentary lifestyle in relation to the level of H-score expression. Shapiro-Wilk, Levene, and Student's t tests were used for alcoholism, smoking, overweight, diabetes mellitus, and arterial hypertension.

Nonparametric Wilcoxon Mann-Whitney tests and Spearman's association coefficient were performed to determine the difference between the two groups assessed: those cases with lymph node metastases and those without lymph node metastases. Finally, the Levene homogeneity test was performed on the basis of the mean.

2.6 Ethical considerations

The study did not represent any risk for the patients, since they were not directly involved, only the histopathological material was available without any additional immediate intervention.

The data were managed by numbers assigned to the material by the institution, maintaining strict confidentiality of the available information. The data were produced in accordance with Mexican Federal Law for the protection of personal data held by individuals.

After reviewing the Official Mexican Standard NOM-004-SSA3-2012 of the Clinical Record and the Declaration of Helsinki, it was determined that there was no risk for the patients involved; finally, approval was obtained from the Ethics and Research Committee of the Dr. Ignacio Morones Prieto Central Hospital, and the registration number 86-22 was assigned.

3. Results

3.1 Population Characteristics

The characteristics of the population and the risk factors found were analyzed according to SOX9 expression and lymph node status. Starting with age, a mean of 60 years at colorectal cancer presentation was obtained; 37 of those

patients were under 60 years of age and 42 were older than 60 years of age. Of the cases with high immunoexpression for SOX9 in patients under 60 years of age, 22 cases were found (10 with lymph node metastasis and 12 without metastasis), while in patients over 60 years of age, there were 17 cases (8 cases with lymph node metastasis and 9 cases without metastasis). As for cases with low expression for SOX9 in patients younger than 60 years, the result was 15 cases (9 cases with lymph node metastasis and 6 cases without metastasis) and 25 cases (11 cases with lymph node metastasis and 14 cases without metastasis) in patients older than 60 years. As a point of interest, 6 cases of patients younger than 40 years were identified, of which 5 presented high expression for SOX9, with metastasis (n = 3) and without metastasis (n = 2), and one case with low immunoexpression for SOX9 belonging to the group without metastasis.

Regarding gender, 46 of the 79 cases were males and 33 were females. Regarding SOX9 immunoexpression and gender, Table 1 can be consulted.

Table 1. SOX9 immunoexpression and sex

Gender/Group	With metastases		Without metastases		Total (%)
	≥ 145	≤ 144	≥ 145	≤ 144	
Female	8	6	13	6	33 (42)
Male	10	14	8	14	46 (58)
Total	18	20	11	20	79 (100)

Regarding histological type, 70 (88.6%) cases corresponded to adenocarcinoma type NOS (formerly called intestinal), eight (10.1%) cases to mucinous adenocarcinoma and one (1.2%) to adenocarcinoma with signet ring cell pattern.

Regarding tumor location, 43 cases were found in the left colon, and of these, 20 had high SOX9 expression (8 with metastasis and 12 without metastasis); 23 cases presented low SOX9 expression (12 with metastasis and 11 without metastasis). On the other hand, 30 cases were located in the right colon, of which 17 presented high expression for SOX9 (9 with metastasis and 8 without metastasis), while 13 presented low expression (6 with metastasis and 7 without metastasis). In the rectum a total of 4 cases were located, of which 2 presented high expression for SOX9 (one with metastasis and one without metastasis) and in two cases the immunoexpression was low (both cases without metastasis). One case of total colectomy with low immunoexpression for SOX9 and no lymph node metastasis was found. There was one case in which the location of the carcinoma was not specified and which also presented low immunoexpression of the biomarker.

Regarding the degree of differentiation, 14 cases corresponded to well-differentiated colorectal carcinomas, of which 9 showed high SOX9 immunoexpression (3 with lymph node metastasis and 6 without metastasis) and the remaining 5 cases showed low SOX9 expression (all 5 without lymph node metastasis). On the other hand, 52 cases with moderately differentiated grading were obtained and of these, 24 with high SOX9 expression (13 with lymph node metastasis and 11 without metastasis) and 28 with low expression (17 with metastasis and 11 without metastasis). Finally, poorly differentiated carcinoma was present in 13 cases of which 6 expressed high SOX9 (2 with metastasis and 4 without metastasis) and 7 low SOX9 (3 with metastasis and 4 without metastasis).

The reported tumor size had a total mean of 6.13 cm, with the smallest being 1 cm and the largest 17 cm; 2 cases did not report tumor size because it was a review of slides from an external laboratory.

Perforation in colorectal carcinoma was present in 16 of the total 79 cases; of these, 9 had high SOX9 expression (6 with lymph node metastasis and 3 without metastasis) while 7 had low expression (4 with metastasis and 3 without metastasis). The remaining 63 cases did not present perforation and in relation to SOX9 expression, 30 presented with high expression (12 with metastasis and 18 without metastasis) and 33 with low expression (16 with metastasis and 17 without metastasis).

Regarding lymphovascular invasion, of the total population of 79 cases, its presence was reported in 56 cases and its absence in 23 cases; of the 56 cases with invasion, 27 had high SOX9 expression (15 with metastasis and 12 without lymph node metastasis) and 29 had low expression (19 with metastasis and 10 without metastasis). On the other hand, of the 23 cases with absence of invasion, 12 had high expression (3 with metastasis and 9 without metastasis) and 11 had low expression (one with metastasis and 10 without metastasis).

Perineural invasion was observed in 33 cases, of which 15 had high SOX9 expression (9 with lymph node metastases and 6 without metastases) and 18 low expression (11 with metastases and 7 without metastases). The remaining 46 cases (n = 79) had 24 with high SOX9 expression (9 with metastases and 15 without metastases) and 22 with low SOX9 expression (9 with metastases and 13 without metastases).

For surgical edges, 63 cases were found with negative edges, and of these, 30 expressed low SOX9 (10 with lymph node metastases and 20 without metastases) and 33 expressed high SOX9 (16 with metastases and 17 without metastases). One case was reported with positive distal and radial surgical edges, which expressed low SOX9 and had lymph node metastases. With positive proximal and radial edges, one case was found, which had high SOX9 expression and lymph node metastasis. The remaining 14 cases were reported with positive radial border, and of these, 8 had high SOX9 expression (7 with metastasis and 1 without metastasis) and 6 had low SOX9 expression (3 with metastasis and 3 without metastasis).

The classification of the TNM system was as follows. Initially, in relation to T, the following was obtained: T1 was one case which presented high expression of SOX9 without metastasis to lymph nodes; T2 had a total of 18 cases, of which 8 had high expression (one with metastasis and 7 without metastasis) and 10 with low expression (4 with metastasis and 6 without metastasis); T3 had 34 cases, 19 with high expression (10 with metastasis and 9 without metastasis) and 15 with low expression (8 with metastasis and 7 without metastasis); T4 is subdivided into T4a and T4b, and therefore, for T4a, 17 cases were reported with 8 with high expression (5 with metastasis and 3 without metastasis) and 9 with low expression (4 with metastasis and 5 without metastasis), and T4b had 9 cases, 3 cases with high expression (2 with metastasis and 1 without metastasis) and 6 with low expression (4 with metastasis and 2 without metastasis). The results of N have already been commented previously. As for M, information was found on 11 cases, since during follow-up it was identified that they had histopathologic studies of involvement of other organs due to suspected metastasis and cases with metastasis to the peritoneal surface, leaving 3 cases as M1a, one with high expression (with lymph node metastasis) and 2 with low expression (one with metastasis and one without metastasis), while in M1c there were 8 cases, of which 5 had high SOX9 expression (4 with metastasis and one without metastasis) and 3 with low expression (all with metastasis). The remaining 68 cases were reported as Mx due to lack of information necessary for their categorization. The data described are summarized in Table 2.

Table 2. Population characteristics

		SOX9 immunoexpression	
		High (≥ 145) n = 39	Low (n ≤ 144) n = 40
Age (years)	> 60	17	25
	≤ 60	22	15
Sex	Female	21	12
	Male	18	28
Histologic type	NOS	34	36
	Mucinous	5	3
	Signet ring cell	0	1
Location	Sigmoid colon	0	4
	Right colon	17	13
	Left colon	20	19
	Total colectomy	0	1
	Rectum	2	2
	Unspecified laterality of the colon	0	1
Differentiation	Well	9	5
	Moderate	24	28
	Poor	6	7
Perforation	Absent	30	33
	Present	9	7
Lymphovascular invasion	Absent	12	11
	Present	27	29
Perineural invasion	Absent	24	22
	Present	15	18
Surgical margins	Negative	30	33
	Positive distal and radial	0	1
	Positive proximal and radial	1	0
	Positive radial	8	6
TNM system	T1	1	0
	T2	8	10

	T3	19	15
	T4a	8	9
	T4b	3	6
	N0	21	20
	N1a	5	5
	N1b	6	8
	N2a	4	2
	N2b	3	5
	M1a	1	2
	M1c	5	3
	Mx	33	35

Note: Summary of the characteristics of the population with colorectal cancer per group by H-score determination of SOX9 expression.

Another point to be analyzed in this study is the clinical data, such as: treatment prior to surgery, sedentary lifestyle, overweight, smoking, alcoholism, and the chronic degenerative diseases, diabetes mellitus and arterial hypertension. Due to regulatory issues regarding the safekeeping of the hospital's clinical records, only 5 years prior to this study were available; consequently, information was only obtained on 57 cases (n = 57) corresponding to this period of time.

Previous treatment in patients with colorectal carcinoma was reported in 3 of the 57 cases, one case received chemotherapy prior to surgery and the remaining two received concurrent radiotherapy and chemotherapy. Regarding SOX9 expression, two cases had high expression without lymph node metastasis and the remaining case had low expression and presence of lymph node metastasis. The location of the carcinoma of these cases with previous treatment was two cases in the left colon and the remaining one in the rectum. The cases without previous treatment were a total of 54 cases, of which 23 had high SOX9 expression (10 with lymph node metastasis and 13 without metastasis) and 31 with low expression (16 with metastasis and 15 without metastasis). Chi-square test was performed to evaluate H-score variables (high and low) with previous treatment (absent and present) and significance was obtained with a $p = 0.413$.

Sedentary lifestyle was present in 2 cases and absent in 55 cases. The 2 cases did not present lymph node metastasis and in relation to SOX9 expression, one had low and the other high expression. The 55 cases had 24 cases with high expression (10 with metastases and 14 without metastases) and 31 cases with low expression (17 with metastases and 14 without metastases). The relationship between sedentary lifestyle and SOX9 was evaluated by means of chi-square, obtaining a $p = 0.859$.

Smoking had 7 cases with the presence of smoking and 50 cases without. Of the 7 cases, 2 had high SOX9 expression (one with lymph node metastasis and one without metastasis) and 5 had low expression (3 with metastasis and 2 without metastasis). Of the 50 cases, 23 showed high expression (9 with metastases and 14 without metastases) and 27 low expression (14 with metastases and 13 without metastases). The Shapiro-Wilk test was performed, resulting in $p = 0.196$ and 0.933, which indicated that the data have normal distribution. Subsequently, Levene's test was used with a p based on the mean of 0.694, and Student's t-test with a significance (assuming equal variances) of 0.421.

In relation to alcoholism, it was present in 6 cases, while according to the clinical record, the antecedent was denied in 51 cases. Of the 6 cases with alcoholism with SOX9 immunoexpression, 2 had high expression (one with metastasis and one without metastasis) and 4 had low expression (3 with metastasis and one without metastasis). Of the 51 cases, 23 had high SOX9 expression (9 with lymph node metastasis and 14 without metastasis) and 28 had low expression (14 with metastasis and 14 without metastasis). The Shapiro-Wilk test gave a p = 0.225 and 0.148. Levene's test based on the mean gave a p = 0.958 and Student's t-test gave a p = 0.389.

For overweight, 7 cases were found with presence and 50 cases with absence; of the 7 cases, 2 had high SOX9 expression (both with lymph node metastasis) and 5 had low expression (4 with lymph node metastasis and one without metastasis). Of the 50 cases, 23 had high SOX9 expression (8 with metastases and 15 without metastases) and 27 cases had low expression (13 with metastases and 14 without metastases). The Shapiro-Wilk result was a p = 0.178 and 0.578. Levene's test based on the mean was 0.320 and Student's t test gave a p = 0.489.

Arterial hypertension was present in 14 cases and absent in 43 cases; of the 14 cases, 5 had high SOX9 expression (4 with lymph node metastasis and 1 without metastasis) and 9 had low expression (5 with metastasis and 4 without metastasis). Of the 43 cases, 20 had high SOX9 expression (6 with metastases and 14 without metastases) and 23 had low expression (12 with metastases and 11 without metastases). Shapiro-Wilk gave a p = 0.318 and 0.546. Levene's test resulted in 0.235 and Student's t test in 0.230.

Diabetes mellitus was present in 12 cases and absent in 45 cases; in the 12 cases with diabetes mellitus, 6 were found with high SOX9 expression (4 with metastasis and 2 without metastasis) and 6 with low expression (4 with metastasis and 2 without metastasis), while of the 45 cases with absence of diabetes mellitus, 19 had high SOX9 expression (6 with metastasis and 13 without metastasis) and 26 had low expression (13 with metastasis and 13 without metastasis). The Shapiro-Wilk test gave a p = 0.434 and 0.725. Levene's test based on the mean gave a p = 0.468 and Student's t test a p = 0.766.

3.2 SOX9 immunoexpression and lymph node metastasis

Of the 79 cases studied, 2 groups were obtained: the group with lymph node metastases (n = 38) and the group without lymph node metastases (n = 41). The nuclear immunoexpression of SOX9 measured by H-Score is shown in Figure 1.

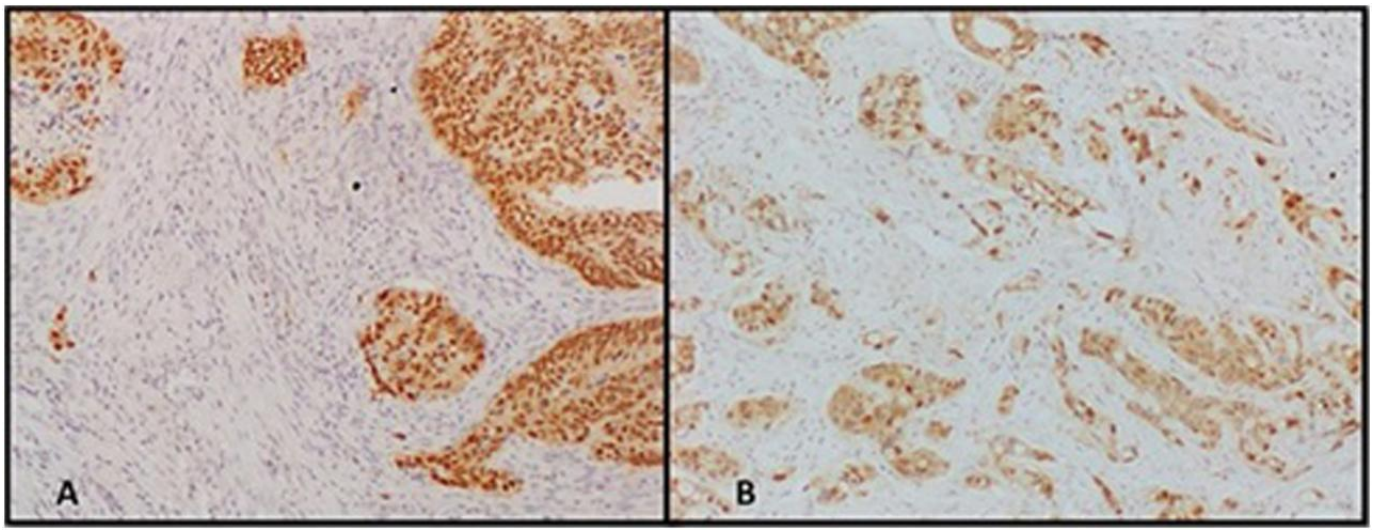

Figure 1. SOX9 scores by H-score. Nuclear staining of SOX9 in colon with high and low expression by H-score. A. High nuclear expression of SOX9 (H-score ≥ 145). B. Low nuclear expression of SOX9 (H-score < 144). The shown is a photomicrograph of the colon, in which nuclear expression of SOX9 antibody is observed. Image A corresponds to an example of high nuclear labeling and image B to low nuclear expression.

With respect to high SOX9 immunoexpression, the following was obtained: 18 cases (47%) corresponded to the group with lymph node metastases and 21 cases (51%) to the group without metastases, for a total of 39 cases (Figure 2).

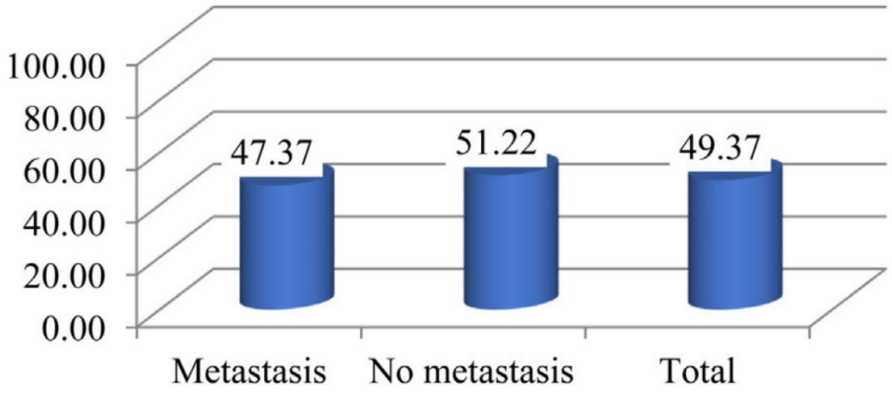

Figure 2. Percentage distribution of cases with high SOX9 immunoexpression in the group with lymph node metastases (47%) and the group without lymph node metastases (51%). Bar graph of cases with high SOX9 immunoexpression divided into the group with lymph node metastases and the group without lymph node metastases is shown.

The 40 cases that had low SOX9 expression were 20 cases with metastases and 20 cases without metastases. Finally, with the results of both groups, a p = 0.73 was obtained, as shown in Table 3.

Table 3. Results of H-score groups and lymph node status

Lymph node metastasis	SOX-9		Total	% ≥ 145
	≥ 145	≤ 144		
Yes	18	20	38	47.37
No	21	20	41	51.22
Total	39	40	79	49.37
	χ^2 =	0.117	p =	0.73

Note: High and low SOX9 immunoexpression results related to lymph node status and the p value.

The distribution of H-score values of cases with high SOX9 expression presented a similar trajectory, as shown in Figure 3.

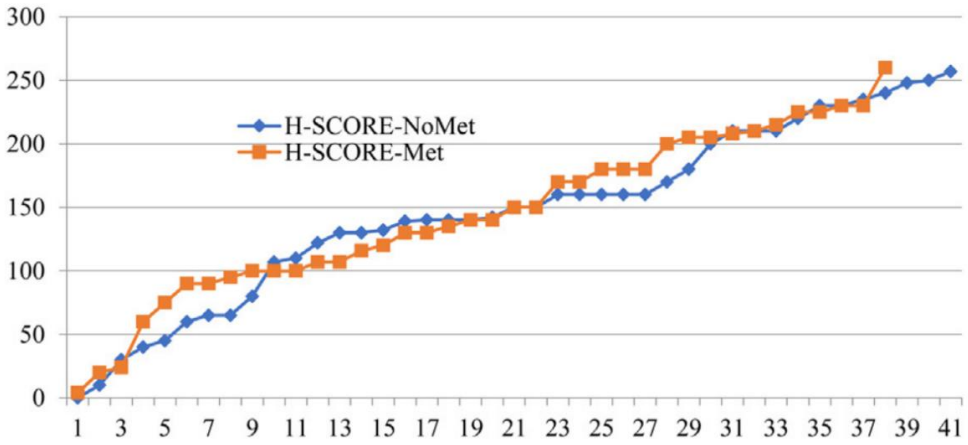

Figure 3. Distribution of H-score values. Ordered distribution of high SOX9 immunoexpression values by H-score (≥ 145) in the groups with and without lymph node metastases. It can be seen how both the group with metastases (orange line) to lymph nodes and the group without metastases (blue line) had a very similar distribution of values.

Based on the N of the TMN staging system, 41 cases were N0, while 38 cases presenting lymph node metastasis were classified as follows: 10 cases N1a, of which 5 had high expression and 5 had low expression; with N1b there were 14 cases, 6 with high expression and 8 with low expression; on the other hand, 6 cases were obtained with N2a, of which 4 had high expression and 2 had low expression; and finally, 8 cases were N2b, of which 3 had high expression and 5 had low expression of SOX9.

3.3 Non-parametric tests

Spearman's association coefficient and Wilcoxon Mann-Whitney tests were performed to determine the difference between the 2 groups assessed. Spearman's association coefficient gave a p = 0.73 and a correlation coefficient of -0.038 (Table 4).

Table 4. Spearman's rho

			H-score	Metastasis
Spearman's rho	H-score	Correlation coefficient	1.000	−0.038
		Significance (bilateral)		0.736
		n	79	79
	Metastasis	Correlation coefficient	−0.038	1.000
		Significance (bilateral)	0.736	
		n	79	79

Note: Spearman's rho correlation between the H-score and lymph node metastasis.

The result of the Wilcoxon Mann-Whitney test was a p = 0.67, with a standard deviation of 101.848 (Table 5).

Table 5. Wilcoxon Mann-Whitney test

	n	Rank sum	
	41	1,684	H-score-No metastasis
	38	1,476	H-score-Metastasis
	79	3,160	Total
101.848	Standard deviation		
0.432	z corrected for ties		
0.67	p value (two-tailed)		

Note: Wilcoxon Mann-Whitney test per H-score group, with and without lymph node metastases.

Levene's homogeneity test was performed based on the variances presented previously, resulting in a p = 0.868, as shown in Table 6.

Table 6. The Levene's test for homogeneity of variance

		Levene's statistic	df1	df2	Significance
H-score	Based on the mean	0.028	1	77	0.868

Note: The Levene's test for homogeneity of variance based on the mean, with a p of 0.868, indicating that the variances are not significantly different from each other.

4. Discussion

Colorectal carcinoma is a neoplasm with a high incidence and mortality rate, for which we have sought to identify prognostic factors that guide treatment and the development of new therapies. There are several prognostic factors for colorectal carcinoma; among these is the presence of lymph node metastasis. Proposals have been described in the literature to predict lymph node metastasis through different tools, such as molecular biology and cell culture, to the use of artificial intelligence to fulfill the purpose and thus detect carcinoma at earlier stages [7, 9, 11, 14, 15].

As mentioned above, SOX9 is a transcription factor that has been implicated in stem cell maintenance by participating in tissue homeostasis, regeneration and tumor initiation, and is overexpressed in several tumors, including colorectal carcinoma. Likewise, 5 to 10% of cases present mutation of the gene, and although SOX9 has a dose-dependent effect in intestinal epithelial cells, in metastases it seems to act independently of the presence of the gene; studies have been carried out in cell cultures [20, 21], with other molecular pathology techniques, such as immunohistochemistry, but nothing has been described so far.

The limitations of the study were the small sample size, which was single-center and retrospective. One reason to consider was the COVID-19 pandemic, which paralyzed the care of many patients and their clinical and surgical treatment, resulting in a low number of surgical specimens being received in the pathology department. It was not possible to study the presence of microsatellite instability as an additional variable in this study, since the necessary supplies were not available.

Regarding the characteristics of the population and risk factors, it was determined that the left colon was the most common site of colorectal carcinoma. The mean age of the patients was 60 years, with a higher incidence in those above this age. The male sex was the most affected (46 of the total 79 cases: 58%), and low SOX9 expression prevailed in this segment. These observations are in line with what has been previously reported in the literature. All of the above is in agreement with the frequency reported in the literature.

Intestinal perforation of the carcinoma was present in the minority of cases (16 of 79 cases: 20%), which raises the risk of tumor progression and dissemination. Lymphovascular invasion was found more frequently than perineural invasion. Surgical margins were reported to be negative in most cases. From the TNM system, the most frequent T was T3 due to tumor size. On the other hand, in the N category, 41 cases were reported with N0 classification (negative cases), in contrast to the 38 positive cases, of which N1b was the most recurrent. For M, the lack of clinical data on metastasis was a limitation, since only cases in which the distant organ biopsy was sent to pathology were recorded, and there were few cases in which the information was provided.

Due to the NOM-004-SSA3-2012 clinical record regulations, data on treatment prior to the surgical procedure and personal history of alcoholism, smoking, sedentary lifestyle, overweight, diabetes mellitus and arterial hypertension were only found up to 5 years ago. However, it was possible to conclude that most of the cases did not receive previous treatment, since most of them were admitted with acute abdominal data requiring immediate surgical management.

5. Conclusions

There was no correlation between SOX9 immunoexpression and the presence of metastasis by obtaining a $p = 0.73$, indicating that the presence and/or absence of lymph node metastasis is independent of SOX9 immunoexpression. There was no statistical difference between the group with metastasis and the group without metastasis with SOX9 immunoexpression. Likewise, SOX9 immunoexpression was independent of the histopathologic characteristics of colorectal cancer, its histologic grade, and its stratification according to the pTNM. There was no relation between SOX9 and the clinical data analyzed in the study population. These results were validated by a p-value with no statistical

significance, obtained from the chi-square test and Student's t-test. However, further research studies need to be carried out.

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Conflicts of Interest

The author declares no conflicts of interest regarding the publication of this paper.

References

[1] Nagtegaal I, Arends MJ, Salto-Tellez M, et al. Colorectal adenocarcinoma. In: Odze R, editor. Who Classification of Tumours: Digestive System Tumours. 51st ed. Lyon, France: World Health Organization; 2019. p. 179-87, http://dx.doi.org/10.1111/his.13975

[2] International Agency for Research on Cancer. Colorectal cancer. GLOBOCAN 2020 [Internet]. Lyon: IARC; 2022. 8-Colon-fact-sheet.pdf.

[3] Burgart LJ, Chopp WV, Jain D. Protocol for the examination of resection specimens from patients with primary carcinoma of the colon and rectum. Coll Am Pathol [Internet]. 2022. https://documents.cap.org/protocols/ColoRectal 4.2.0.2 .REL CAPCP.pdf?

[4] Jessup M, Goldberg RM, Asare EA, et al. Colon and Rectum. In: Amin MB, editor. AJCC Cancer Staging Manual. 8th ed New York: Springer; 2017. p. 251-74.

[5] Nguyen L, Goel A, Chung D. Pathways of colorectal carcinogenesis. Gastroenterology. 2020;158:291-302, http://dx.doi.org/10 .1053/j.gastro.2019.08.059

[6] Takamatsu M, Yamamoto N, Kawachi H, et al. Prediction of early colorectal cancer metastasis by machine learning using digital slide images. Comput Methods Programs Biomed. 2019;178:155-61, http://dx.doi.org/10.1016/j.cmpb. 2019.06.022.

[7] Ulintz PJ, Greenson JK, Fearon ER, et al. Lymph node metastases in colon cancer are polyclonal. Clin Cancer Res. 2018;24:2214-24, http://dx.doi.org/10.1158/1078- 0432.CCR-17-1425.

[8] Kwak MS, Lee HH, Yang JM, et al. Deep convolutional neural network-based lymph node metastasis prediction for colon cancer using histopathological images. Front Oncol. 2021;13(10):619803, http://dx.doi.org/10.3389/fonc. 2020.619803

[9] Johncilla M, Yantiss RK. Histology of colorectal carcinoma proven and purported prognostic factor. Surg Pathol Clin. 2020;13:503- 20, http://dx.doi.org/10.1016/j.path.2020 .05.008

[10] Aguilar-Medina M, Avedano-Félix M, Lizárraga-Verdugo E, et al. SOX9 stem-cell factor: clinical and functional relevance in cancer. J Oncol. 2019;1:6754040, http://dx.doi.org/10.1155/2019/6754040

[11] Liang X, Duronio GN, Yaying Y, et al. An enhancer-driven stem cell-like program mediated by SOX9 blocks intestinal differentiation in colorectal cancer. Gastroenterology. 2022;162:209-22, http://dx.doi.org/10.1053/j.gastro.2021 .09.044

[12] Lü B, Fand Y, Xu J, et al. Analysis of SOX9 expression in colorectal cancer. Am J Clin Pathol. 2008;130:897-904, http://dx.doi.org/10.1309/AJCPW1W8GJBQGCNI

[13] Contreras L, Gutiérrez MC, Hernández HG. Evaluación de la asociación entre la inmunoexpresión de SOX9 y el grado de diferenciación en carcinoma colorrectal en pacientes del Hospital Central "Dr. Ignacio Morones Prieto". [Tesis]. San Luis Potosí: Universidad Autónoma de San Luis Potosí [Internet].; 2022, https://repositorioinstitucional.uaslp.mx/xmlui/handle/i/7487

[14] Zhou T, Wu L, Ma N, et al. SOX9-activated FARSAAS1 predetermines cell growth, stemness, and metastasis in colorectal cancer through upregulating FARSA and SOX9. Cell Death Dis. 2020;11:1071, http://dx.doi.org/10 .1038/s41419-020-03273-4.

[15] Xu Y, Zhang X, Hu X, et al. The effects of lncRNA MALAT1 on proliferation, invasion and migration in colorectal cancer through regulating SOX9. Mol Med. 2018;24:52, http://dx.doi.org/10.1186/s10020-018-0050-5

[16] Qian Y, Xia S, Feng Z. SOX9 mediated transcriptional activation of FOXK2 is critical for colorectal cancer cells proliferation. Biochem Biophys Res Commun. 2017;483:475-81, http://dx.doi.org/10.1016/j.bbrc.2016.12.119

[17] Xia S, Yang Z, Qi X, et al. Overexpression of SOX9 and DNMT1 predicts poor prognosis and chemoresistance of colorectal cancer. Int J Clin Exp Pathol. 2016;9:589- 600, ijcep0020029.pdf.

[18] Xu C, Ding Y-H, Wang K, et al. Claudin-7 deficiency promotes stemness properties in colorectal cancer through SOX9-mediated Wnt/β-catenin signalling. J Transl Med. 2021;19:311, http://dx.doi.org/10.1186/s12967-021-02983-3

[19] Liang J, Liu Q, Xia L, et al. Rac1 promotes the reprogramming of glucose metabolism and the growth of colon cancer cells through upregulating SOX9. Cancer Sci. 2023;114:822-36, http://dx.doi.org/10.1111/cas.15652

[20] Miao D, Wang Y, Jia Y, et al. ZRANB1 enhances stemcell-like features and accelerates tumor progression by regulating SOX9-mediated USP22/Wnt/β-catenin pathway in colorectal cancer. Cell Signal. 2022;90:110200, http://dx.doi.org/10.1016/j.cellsig.2021.110200

[21] Carrasco-Garcia E, López L, Aldaz P, et al. SOX9-regulated cell plasticity in colorectal metastasis is attenuated by rapamycin. Sci Rep. 2016;6:32350, http://dx.doi.org/10.1038/srep32350

[22] Candy PA, Phillips MR, Redfern AD, et al. Notch-induced transcription factors are predictive of survival and 5-fluorouracil response in colorectal cancer patients. Br J Cancer 2013. 2013;109:1023-30, http://dx.doi.org/10.1038/bjc.2013.431

[23] Roche KC, Gracz AD, Liu XF, et al. SOX9 maintains reserve stem cells and preserves radio resistance in mouse small intestine. Gastroenterology. 2015;149:1553-63, http://dx.doi.org/10.1053/j.gastro.2015.07.004

[24] Fitzgibbons P, Connolloy J. Template for reporting results of biomarker testing of specimens from patients with carcinoma of the breast. Coll Am Pathol [Internet]. https://www.cap.org/protocols-and-guidelines/cancer-reporting-tools/cancer-protocol templates

[25] Thabane L, Ma J, Chu R, et al. A tutorial on pilot studies: the what, why and how. BMC Med Res Methodol. 2010;10:1, http://dx.doi.org/10.1186/1471-2288-10-1

[26] Fernández P. Determinación del tamano muestral. Fisterra [Internet]. https://www.fisterra.com/formacion/metodologia-investigacion /determinacion-tamano-muestral/#sec2

[27] Hérnandez-Samperi R. Análisis de los datos en la ruta cuantitativa. In: Hernández- Samperi R, editor. Metodología de la investigación las rutas cuantitativa, cualitativa y mixta. Ciudad de México: Mc Graw Hill; 2018. p. 363-9.