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Immunohistochemical analysis of the expression of ALCAM and ALDH1 markers in patients with colorretal adenocarcinoma and association with clinicopathological outcomes

Análise imunoistoquímica da expressão dos marcadores ALCAM e ALDH1 em pacientes com adenocarcinoma colorretal e associação com desfechos clinicopatológicos

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ABSTRACT

Background: Colorectal cancer has a high global mortality and tumor markers have emerged as diagnostic, management and prognostic indicators. New markers are being studied.

Objective: To verify if there is a correlation between the immunohistochemical expression of ALCAM and ALDH1 proteins in colorectal adenocarcinoma tissue with epidemiological and clinicopathological characteristics, in particular their impact on disease progression and death.

Method: Observational, single-center, analytical, retrospective study, through the investigation of patients undergoing surgical resection for colorectal cancer.

Result: 122 patients were evaluated. Regarding progression, it was shown that in individuals with positive ALCAM (n=40), 14/40 (35%) had progression, and for positive ALDH (n = 54), 22/54 (40.7%). For death, the analysis of ALCAM positive (n = 40), 24/40 (60%) died, and ALDH1 positive (n = 54), 33/54 (61.1%).

Conclusion: The immunohistochemical expression of ALCAM and ALDH1 markers was not associated with disease progression and death; it was also not possible to observe a correspondence relationship with the evaluated markers.

KEYWORDS: Colorectal cancer. ALCAM. ALDH1

RESUMO

Introdução: O câncer colorretal apresenta alta mortalidade global e marcadores tumorais têm surgido como sinalizadores de diagnóstico, manejo e prognóstico. Novos marcadores estão sendo estudados.

Objetivo: Verificar se há correlação da expressão por imunoistoquímica das proteínas ALCAM e ALDH1 em tecido com adenocarcinoma colorretal com as características epidemiológicas e clinicopatológicas, em particular o seu impacto na progressão de doença e no óbito.

Método: Estudo observacional, unicêntrico, analítico, retrospectivo, através da investigação de pacientes submetidos à ressecção cirúrgica por câncer colorretal.

Resultado: Foram avaliados 122 pacientes. Em relação a progressão, mostrou-se que nos indivíduos com ALCAM positiva (n = 40), 14/40 (35%) tiveram progressão, e para ALDH positiva (n = 54), 22/54 (40,7%). Para óbito, a análise da ALCAM positiva (n = 40), 24/40 (60%) morreram, e ALDH1 positivo (n = 54), 33/54 (61,1%).

Conclusão: A expressão imunoistoquímica dos marcadores ALCAM e ALDH1 não apresentou associação com a progressão de doença e óbito; também não foi possível observar relação de correspondência com os marcadores avaliados.

PALAVRAS-CHAVE: Câncer colorretal. ALCAM. ALDH1.

Central Message

Determining the stage of progress, extent, and severity of a tumor at the time of diagnosis is essential to establish the treatment strategy and to estimate the evolution of the disease. Thus, studying the correlation of immunohistochemical expression of ALCAM and ALDH1 proteins in tissue with colorectal adenocarcinoma may help impact disease progression and death.

Perspective

Although initial studies have pointed to ALCAM and ALDH1 as potential prognostic markers in colorectal cancer, there are still several conflicting points such as: 1) the event of disease progression in isolation; 2) death, also evaluated in isolation, is more present in patients with primary rectal tumors; 3) in lung metastasis, clinical stage and ALCAM marker were statistically significant, which did not occur with ALDH1. To minimize these points, it is necessary to refine the research of these substances, quantitatively and qualitatively, to ensure more conclusive results.

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INTRODUCTION

Colorectal cancer (CRC) is the third most common cause of cancer death in the world (mortality 8.9%). The pillars of its treatment consist of surgical procedure, chemotherapy and radiotherapy. Although surgical procedure can be potentially curative, less than 25% of cases are operable with recurrence rates of up to 70%. Inoperable tumors, relapses, or metastatic tumors are usually treated by palliative chemotherapy.[1] Despite these, the prognosis of metastatic tumors remains poor.[2]

Determining the stage of progression (stage), extent, and severity of the tumor at the time of diagnosis is essential to establish the treatment strategy and to estimate the evolution of the disease. The classifications used to define its stage is: degree of cell differentiation, clinical and pathological stages, lymph node involvement, and presence of distant metastasis.[3]

Colorectal cancer restricted to the wall of the intestine (stages I and II) is potentially curable due to early detection and treatment. It has a 5-year survival between 70-90%. However, most countries do not have a screening program that allows for their early detection.[4] In contrast, the median 5-year survival in regional (stage III) and distant (metastatic; stage IV) is approximately 50-70% and 10-14%, respectively.[4] These rates are mainly attributed to the disruption of the intestinal wall by the tumor and its lymphatic dissemination to distant organs through the bloodstream. The incidence of CRC increases after 50 years of age, with 90% of cases being within this age group.[4,5]

Thus, the aim of this study was to correlate the immunohistochemical expression of ALCAM and ALDH1 proteins in tissue with colorectal adenocarcinoma with the epidemiological and clinicopathologic characteristics of the patients, in particular their impact on disease progression and death.

METHOD

This research was carried out in the Graduate Program in Principles of Surgery of the Mackenzie Evangelical College of Paraná, in partnership with Brigham and Women's Hospital, Harvard Medical School Molecular Oncology and Immunology and Renal Division, Boston, USA. It was approved by the Research Ethics Committee of the Evangelical Beneficent Society of Curitiba, under opinion CAAE 66365117.0.0000.0103, in accordance with the precepts of Resolution 466/12 of the National Health Council/Ministry of Health (CNS/MS). It is an observational, single-center, analytical, and retrospective study.

Patients from the Evangelical Mackenzie University Hospital, Curitiba, PR, Brazil, who underwent surgical resection for colorectal cancer and whose medical records were accessible for consultation, were studied. Likewise, it was necessary to have paraffin blocks available that confirmed the diagnosis of colorectal adenocarcinoma and, also, to be submitted to new divisions. Patients under 18 years of age, with a diagnosis of non-adenocarcinoma colorectal cancer, impediments regarding clinical-epidemiological information and, also, infeasibility of obtaining these paraffin blocks (due to damage due to time or poor sampling) were excluded.

The initial sample consisted of 219 patients. Of this total, 29 did not have the paraffin block, 32 blocks had a very small sample of adenocarcinoma biopsy, which made fractionation unfeasible; 5 were diagnosed with non-adenocarcinoma colorectal cancer; 19 had incomplete clinical-epidemiological data; and 12 could not use the block. Thus, the final sample consisted of 122 individuals diagnosed with colorectal adenocarcinoma and with clinical-epidemiological information and complete blocks available.

The selection was carried out through the screening of the hospital's electronic medical records (PAGU system) using ICDs C18, C19, C20 and C21. The acceptance criterion for the block to be chosen was defined by the minimum need for a tumor sample that would allow additional sections without the material being fully used. Next, the respective slides were reviewed by a pathologist. This stage was considered as the second evaluation, since the first was carried out by a specialist who issued the first report. The study selected the blocks that presented the greatest tumor mass in cases in which there was more than 1 block to be reviewed and made new H&E slides for cases in which the slides were not found, to confirm adenocarcinoma. Thus, these pertinent and appropriate blocks were referred to immunostaining with ALCAM and ALDH.

For the research of clinical-epidemiological data, in addition to the electronic medical record system, telephone contact was made with patients or their families in cases of acquisition of extra information. The information was distributed in an Excel® table according to the following pattern: name, date of birth, age at diagnosis, gender, contact telephone number, paraffin block numbering, histological diagnosis, site of primary tumor, ICD-10, date of diagnosis, pathological TNM classification, site of metastasis at diagnosis (if present), TNM classification according to the AJCC/UICC of the Tumor Staging Manual 6th edition, surgical resection status (R), disease progression (if present), progression-free time (if present), date of progression (if present), site of progression (if present), survival time in months, event death (if present), follow-up time, date of death (if present), last outpatient visit (if present), and finally, result of ALCAM and ALDH immunostaining.

As variables, age at diagnosis was considered, the age group was tabulated by decades and, in order to improve the comparison, the following decades were grouped: under 50 years old, between 50-65 years old and over 65 years old. Regarding gender, they were classified as male and female, as reported in their medical records. The topography was in accordance with information from the medical record or report of the pathological anatomy in the ascending, transverse, descending, sigmoid and rectal colons. In cases of uncertain precise information, it was classified

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as indeterminate. It was decided to group patients with ascending and transverse colon tumors. Thus, 4 groups were obtained: ascending/transverse colon, descending colon, sigmoid and rectum.

In cases of metastasis at the time of diagnosis, its location was fixed: hepatic, pulmonary, peritoneal, or other sites (different sites of metastasis other than those already contemplated). Patients were classified as clinical stage 0 to IV, according to the classification of the American Joint Commission on Cancer/Union for International Cancer Control (AJCC/UICC), Cancer Staging Manual 6th edition.

Disease progression was documented based on evidence of local recurrence or distant metastasis. Local recurrence referred to patients who already met the criteria for cure and presented disease at the site of resection of the primary disease in their follow-up. Metastases referred to evidence of neoplasia in other sites, such as surgical sites (lymph nodes, peritoneum, contiguous organs) or at a distance (liver, lung, peritoneum or other sites) and were evaluated for their insertion as local or distant disease progression. The time free of the disease was calculated in months, taking into account the period in which the patient remained without signs and symptoms of the disease. Survival time was determined in months and defined as from the time of diagnosis of colorectal adenocarcinoma to the time the patient died. The follow-up time was determined in months and defined as from the moment of diagnosis of colorectal adenocarcinoma until the date of last contact, by outpatient medical consultation or telephone contact. Pathological staging was performed through the pathological anatomy report, which supported the postoperative pathological staging (pTNM) of the colorectal adenocarcinoma lesions used in the research. The 6th edition of the AJCC was maintained as the basis, as used at the time in the hospital.

Regarding the data on the post-surgical resection status, the pathology report was used to define it, observing information regarding resectability and the margins involved. For information on the degree of histopathological tumor differentiation, the pathology report after surgical resection was used, also according to the same edition of the AJCC.

Analysis of tumor biomarkers

It was performed using the tissue microarray (TMA) technique, followed by immunohistochemical labeling. Tissue Tek Quick-ArrayTM was used to make the paraffin blocks, which contained tweezers with diameters of 1-3 mm for the extraction of the desired fragment to be submitted to immunohistochemistry, and the 2 mm tweezers were used in the research.

The following steps were followed in the assembly of the multisample blocks: 1) selection of the areas that contained the greatest representation of the neoplasm on the slides stained in H&E, and marked with an overhead projector pen; 2) marking of this signage on the respective paraffin block, entitled as donor block; 3) an Excel spreadsheet was created with the cases, similar to a Cartesian map with 10 columns and 6 rows, with the first cell being blank because it served as a mark for the beginning of the reading of the slides and in the others the numbers of case records were noted; 4) multisample block made from a paraffin mold containing 60 holes with diameters of 2 mm whose objective was to follow the order listed in the Excel® spreadsheet created; 5) extraction of the marked area of the donor block with TMA equipment; 6) allocation in the corresponding holes in the paraffin mold, following the order created in the Excel® spreadsheet; 7) in determining the starting point of the slide reading, the first cell, corresponding to the first hole of this mold, was destined for tissue not related to the study, in this case a placenta fragment; 8) paraffin embedded mold; 9) 5 µm microtomy with obtained material placed on Superfrost Plus (hydrophilic) slides to initiate immunohistochemical immunostaining.

The immunohistochemistry technique was performed on the Ventana Bench Mark Ultra TM instrument with integrated 3-in-1 processing. In this case, the TMA slides were prepared with deparaffinization, rehydration, antigenic recovery with Cell Conditioning 1 (high pH) and Cell Conditioning 2 (low pH) buffers. The primary antibodies (Table 1) were incubated for 16-20 min at room temperature.

TABLE 1 — Description of the primary antibodies with their respective manufacturers

Biomarker	Primary antibody	Manufacturer
ALCAM	EPR2759 Clone(2)	Medaysis
ALDH	ALDH1A1 clone 44	Medaysis

Next, the slides were submitted to the immunoperoxidase technique, and the amplification of the marking was performed through the ultra View Universal DAB Detection® Kit. After immunostaining, the TMA slides were reported, and positive internal and external controls tested the fidelity of the reactions. The slides were reported on the Olympus CX31 microscope by 2 different pathologists, at different times. The following parameters were used to report the tumors for immunostaining: positive ALCAM due to the presence of labeling on the cytoplasmic membrane and ALDH due to the presence of staining on the cytoplasm; negative, if the antibody was not visualized in any histological distribution; indeterminate, if it was not possible to read the slide due to the poor quality of the sample.

Statistical analysis

The data were analyzed by the Stata/SE v.14.1 computer program. StataCorpLP, USA. The results of quantitative variables were described as means, standard deviations, medians, and minimum and maximum values. Categorical variables were described by frequencies and percentages. For the analysis of factors associated with time to disease progression (PEVENT), Fine and Gray models were adjusted considering death as a competitive risk. After

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adjustment, the estimated measure of association was the subdistribution hazard ratio (SHR). For the survival analysis, Cox regression models were adjusted, and hazard ratio values were estimated. For both models, the Wald test was used to assess the significance of the variables. Values of $p < 0.05$ indicated statistical significance. The data were analyzed with the Stata/SE v.14.1 computer program. Stata Corp LP, USA.

RESULT

The analysis was performed based on data from 122 patients who had valid data for the markers. One tumor was considered in each patient.

Age ranged from 20-91 years, with a mean of 61.[9]. There was a prevalence of disease in the 5th and 6th decades of life (54.9%). Of the 122 evaluated, 63 were men (51.6%) and 59 women (48.4%). The decreasing incidence by topography, according to the classification chosen by the research group, was as follows: 42 cases (35.9%) in the ascending/transverse colon, 31 (26.5%) in the sigmoid, 27 in the rectum (23.1%), 17 (14.5%) in the left colon. In 5 it was not possible to determine the precise location, receiving the indeterminate classification.

It was found that 87 (71.3%) of the patients did not have metastasis at diagnosis and 35 (28.7%) did. The most common site was the liver with 24 cases (19.7%), followed by the peritoneum with 9 (7.4%), lungs with 5 (4.1%) and other sites with 10 (8.2%). At the time of diagnosis of primary cancer, 13 patients had metastasis to more than 1 site.

According to the UICC 6 edition, of the total, 1 (0.8%) was stage 0, 14 (11.5%)

I, 33 (27%) II, 39 (32%) III and 35 (28.7%). Cases with local involvement (stage 0/I/II) accounted for 39.3% (48 cases), while those with lymph node and metastatic involvement (III/IV) accounted for 60.7% (74 cases).

Of the total cases, 43 (35.2%) had recurrence or progression of the neoplasm, while 79 (64.8%) did not, and 14 had disease progression in more than one organ. The most common site of progression was the liver, with 16 cases (13.1%), followed by the peritoneum, with 13 cases (10.7%), and the lung, with 12 (9.8%). Other places totaled 20 (16.4%). There was an increase in patients with disease progression over time. The median survival time estimated by the Kaplan-Meier method was 30 months in the group studied, ranging from 0 to 85.7 months. Of the total of 122 patients, 68 (55.7%) died. In the others, there was a median follow-up of 35.9 months (Table 1). The median survival time of patients who died was 15.[6] months.

TABLE 1 — Follow-up time during the study period

Death	Follow-up death (months)					
	n	Average	Median	Min	Max	DP
No	54	39,9	35,9	0,20	101	27,7
Yes (survival time)	68	18,6	15,6	0	85,7	17,2
Everyone	122	28,0	22,5	0	101	24,8

Regarding pathological staging, there was a predominance of pT3 cases with 71 (58.2%) patients, pN0 with 52 (42.6%) and pMx with 72 cases (59%) patients (Table 2).

TABLE 2 — Distribution of patients according to TNM

pTis	1	0,8
pT1	0	0
pT2	16	13,1
pT3	71	58,2
pT4	28	23
Biopsy	6	4,9
pNx	8	6,6
pN0	52	42,6
pN1	37	30,3
pN2	21	17,2
Biopsy	4	3,3
Pmx	72	59
pM0	13	10,7
pM1	33	27
Biopsy	4	3,3
Total	122	100

Regarding the status of surgical resection, 85 (72%) were classified as R0 (complete resection), 23 (19.5%) as R1 (microscopic residual tumor), and 10 (8.51%) as R2 (macroscopic residual tumor). Of the total sample, 4 could not have their data evaluated in this criterion.

Regarding the degree of differentiation, there was a predominance of moderately differentiated tumors with 101 samples (82.8%), followed by poorly differentiated tumors with 10 (8.2%) and well-differentiated tumors with 8 (6.6%). Of the total, 2.4% were undetermined.

Immunostaining with ALCAM and ALDH1

After histopathological review, TMA preparation and immunohistochemistry reaction of ALCAM and ALDH1, its expression in tissue with colorectal carcinoma was evaluated. ALCAM positivity was 32.8% (n = 40), while ALDH was 44.3% (n = 54, Table 3). No statistical significance was observed in the presence or absence of the ALCAM and ALDH markers in relation to age, patient diagnosis, gender, degree of differentiation, clinical staging, or metastases at any site.

TABLE 3 — Immunostaining with ALCAM and ALDH1

ALCAM	n	%
Negative	54	44,3
Positive	40	32,8
Inconclusive	28	23
ALDH1	**n**	**%**
Negative	41	33,6
Positive	54	44,3
Inconclusive	27	22,1
Total	122	100

Assessment of factors associated with disease progression

The analysis did not show statistical significance for the presence or absence of ALCAM and ALDH marking. There was also no statistical difference when relating the

time of disease progression with age at diagnosis, gender, degree of differentiation, primary tumor topography, clinical staging at diagnosis, presence or absence of metastases, and compromised surgical resection margin (R1 and R2 resection, Table 4).

TABLE 4 — Univariate analysis for the disease progression event

Variable	Classification	n	%	p*	SHR	95% CI
Age in dx	< 50	22	10 (45,5)			
	50 to 65	45	17 (37,8)	0,300	0,67	0,31 – 1,43
	> 65	55	16 (29,1)	0,124	0,54	0,25 – 1,18
Gender	Fem	59	16 (27,1)			
	Men	63	27 (42,9)	0,127	1,63	0,87 – 3,04
Degree of differentiation*	Well difference	8	3 (37,5)			
	Moderate	101	39 (38,6)	0,738	1,19	0,42 – 3,38
	Little difference	10	1 (10,0)	0,360	0,34	0,04 – 3,36
Topography of the primary tumor**	Right/transverse colon	42	12 (28,6)			
	Left colon	17	5 (29,4)	0,681	1,27	0,41 – 3,89
	Rectosigmoid	31	12 (38,7)	0,495	1,31	0,60 – 2,88
	Straight	27	12 (44,4)	0,332	1,47	0,68 – 3,19
Lung metastasis	No	117	39 (33,3)			
	Yes	5	4 (80,0)	0,019	4,33	1,27 – 14,7
Liver metastasis	No	98	34 (34,7)			
	Yes	24	9 (37,5)	0,557	1,26	0,58 – 2,72
Peritoneal metastasis	No	113	40 (35,4)			
	Yes	9	3 (33,3)	0,806	0,86	0,27 – 2,80
Metastasis to other sites	No	112	41 (36,6)			
	Yes	10	2 (20,0)	0,531	0,64	0,16 – 2,59
Presence of metastasis	No	87	30 (34,5)			
	Yes	35	13 (37,1)	0,360	1,37	0,70 – 2,69
UICC	0/I	15	5 (33,3)			
	II	33	10 (30,3)	0,572	0,75	0,28 – 2,00
	III	39	16 (41,0)	0,394	1,50	0,59 – 3,85
	IV	35	12 (34,3)	0,662	1,25	0,46 – 3,44
UICC grouped	0/I/II	48	15 (31,2)			
	III/IV	74	28 (37,8)	0,085	1,68	0,93 – 3,05
Surgical resection status	R0	85	31 (36,5)			
	R1	23	8 (34,8)	0,739	1,14	0,53 – 2,48
	R2	10	3 (30,0)	0,944	0,95	0,26 – 3,55
Treatment	Operation	105	30 (28,6)			
	Other	17	13 (76,5)	<0.001	3,30	1,88 – 5,79
ALCAM	Negative	54	20 (37,0)			
	Positive	40	14 (35,0)	0,607	0,84	0,43 – 1,65
	Inconclusive	28	9 (32,1)	0,938	0,97	0,44 – 2,12
ALDH1	Negative	41	10 (24,4)			
	Positive	54	22 (40,7)	0,182	1,64	0,79 – 3,40
	Inconclusive	27	11 (40,7)	0,060	2,30	0,97 – 5,50

SHR = subdistribution hazard ratio; 95%CI = 95% confidence interval; Fine and Gray model and Wald test, p < 0.05; * = 3 patients who had an indeterminate degree of differentiation were excluded; ** = excluded 5 patients who had indeterminate topography

However, when relating the time of disease progression with the treatment instituted, it was observed that patients undergoing non-surgical treatment (SHR 3.30; 1.88 – 5.79) had lower survival (p < 0.001). A worse prognosis was also observed when there was metastatic lung disease (SHR 4.33; 1.27–14.7, p < 0.05). The evaluation of disease progression according to immunostaining did not show statistical relevance. The univariate analysis showed that in individuals with positive ALCM (n = 40), 14/40 (35%) had progression, p = 0.607, SHR 0.84 (CI: 0.43-1.65) and for positive ALDH (n = 54), 22/54 (40.7%) had progression, p = 0.182, SHR 1.64 (CI: 0.79-3.40). The evaluation of disease progression according to the clustered UICC stage did not show statistical relevance.

Evaluation of factors associated with death

In this evaluation, no statistical significance was found between the presence or absence of marking of the markers studied. The univariate analysis showed individuals with positive ALCM (n = 40), 24/40 (60%), p = 0.789, SHR 0.93 (CI: 0.54-1.60) to die, unlike positive ALDH1 (n = 54), 33/54 (61.1%), p = 0771, SHR 1.09 (CI: 0.62-1.90).

However, those with poorly differentiated tumors (HR 17.6; 3.5 – 88.6), advanced clinical stage (UICC III/IV, HR 2.52; 1.49 – 4.25), and disease progression event (HR 5.91; 3.37 – 10.4) showed a greater relationship with death (p < 0.001). In cases with primary rectal tumors (HR 2.25; 1.13 – 4.48), liver metastasis at diagnosis (HR 2.02; 1.17 – 3.47) and compromised surgical resection margin (R1 resection, HR 2.00; 1.08 – 3.70), this association was also observed, with statistical significance (p < 0.05, Table 5).

TABLE 5 — Evaluation of factors associated with death

Variável	Classif	n	% de óbitos	p*	HR	IC 95%
Idade no dx	< 50	22	9 (40,9%)			
	50 a 65	45	26 (57,8%)	0,587	1,23	0,58 – 2,64
	> 65	55	33 (60,0%)	0,110	1,83	0,87 – 3,84
Gênero	Masculino	63	32 (50,8%)			
	Feminino	59	36 (61,0%)	0,134	1,44	0,89 - 2,34
Grau de diferenciação*	Bem diferenc	8	2 (25,0%)			
	Moderado	101	58 (57,4%)	0,078	3,55	0,87 – 14,6
	Pouco diferenc	10	7 (70,0%)	0,001	17,6	3,5 – 88,6
Topografia tumor primário**	Retossigmoide	31	13 (41,9%)			
	Cólon direito/transverso	42	19 (45,2%)	0,556	1,24	0,61 - 2,51
	Cólon esquerdo	17	11 (64,7%)	0,125	1,88	0,84 - 4,20
	Reto	27	22 (81,5%)	0,021	2,25	1,13 - 4,48
Metástase pulmonar	Não	117	65 (55,6%)			
	Sim	5	3 (60,0%)	0,714	1,24	0,39 – 3,98
Metástase hepática	Não	98	50 (51,0%)			
	Sim	24	18 (75,0%)	0,011	2,02	1,17 - 3,47
Metástase peritoneal	Não	113	62 (54,9%)			
	Sim	9	6 (66,7%)	0,512	1,32	0,57 - 3,07
Metástases em outros sítios	Não	112	62 (55,4%)			
	Sim	10	6 (60,0%)	0,075	2,17	0,92 – 5,11
Presença de metástase	Não	87	44 (50,6%)			
	Sim	35	24 (68,6%)	**0,004**	2,12	1,27 – 3,52
UICC	0/I	15	7 (46,7%)			
	II	33	15 (45,4%)	0,616	0,79	0,32 – 1,96
	III	39	23 (59,0%)	0,114	1,98	0,85 – 4,64
	IV	35	23 (65,7%)	0,052	2,33	0,99 – 5,46
UICC agrupado	0/I/II	48	22 (45,8%)			
	III/IV	74	46 (62,2%)	0,001	2,52	1,49 - 4,25
Status de ressecção cirúrgica	R0	85	42 (49,4%)			
	R1	23	14 (60,9%)	0,028	2,00	1,08 - 3,70
	R2	10	8 (80,0%)	0,103	1,58	0,88 – 4,02
Tratamento	Cirurgia	105	57 (54,3%)			
	Outros	17	11 (64,7%)	0,527	1,23	0,64 - 2,36
Evento de progressão	Não	79	41 (51,9)			
	Sim	43	27 (62,8)	<0,001	5,91	3,37 – 10,4
ALCAM3	Negative (ref)	54	31 (57,4)			
	Positive	40	24 (60,0)	0,789	0,93	0,54 – 1,60
	Inconclusivo	28	13 (46,4)	0,710	1,13	0,59 – 2,19
ALDH1	Negative (ref)	41	20 (48,8)			
	Positive	54	33 (61,1)	0,771	1,09	0,62 – 1,90
	Inconclusivo	27	15 (55,6)	0,102	1,76	0,89 – 3,48

* = Cox regression model and Wald test, p < 0.05; the variable Progression event was included as time-dependent; * = 3 patients who had an indeterminate degree of differentiation were excluded; ** = 5 patients who had indeterminate topography were excluded.

Evaluation of factors associated with follow-up time and survival

This association showed a trend of the median follow-up higher in earlier clinical stages (0/I/II, Table 6).

TABLE 6 — Follow-up time

Follow-up time (months)						
UICC	n	Average	Median	Minimum	Maximum	Standard deviation
0 / I	15	31,9	30,1	1,37	63,6	17,7
II	33	44,2	47,5	0,03	101	30,5
III	39	20,5	15,6	0	83,2	18,5
IV	35	19,4	15,6	0,03	85,7	20,0

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The 5-year survival rate for stage 0/I/II was 48.5%, and 23.2% for stages III/IV (Table 7). Of the group followed, 68 patients (55.7%) died.

TABLE 7 — Overall survival time and classification according to clinical staging

Survival percentage			
Time	0 / I / II	III / IV	General
Diagnosis	100%	100%	100%
1 year	85,00%	70,20%	76,20%
2 years	80,60%	45,40%	60,60%
3 years	59,60%	33,00%	44,40%
5 years	48,50%	23,20%	34,40%

Assessment of factors associated with biomarkers

In this sample, 16 cases of local disease (stage 0, I, II) were positive, and of these, 5 (31.2%) died. In cases of lymph node or distant metastasis (stages III and IV), 24 cases were positive for ALCAM, and 19 (79.2%) of them died. Despite the relevant values, these data did not obtain statistical significance (Table 8).

TABLE 8 — Relationship between ALCAM marker expression and clinical stage

ALCAM	Classif	n	% of deaths	p*	HR	95% CI
0/I and II	Negative	24	13 (54,2%)			
	Positive	16	5 (31,2%)	0,056	0,36	0,13 – 1,03
	Inconclusive	8	4 (50,0%)	0,938	1,05	0,34 – 3,23
III and IV	Negative	30	18 (60,0%)			
	Positive	24	19 (79,2%)	0,290	1,43	0,74 – 2,79
	Inconclusive	20	9 (45,0%)	0,879	1,07	0,47 – 2,42

* = Cox regression model and Wald test, $p < 0.05$

The ALDH1 sample was positive in 27 cases of local disease (stage 0, I, II), of which 20 (74.1%) died. In cases of lymph node or distant metastasis (stages III and IV), 27 cases were ALDH1-positive, and 12 (48.1%) died. Despite the relevant values, these data did not obtain statistical significance. The inconclusive was relevant (Table 9).

TABLE 9 — Relationship between ALDH1 marker expression and clinical stage

ALDH1	Classif	n	% of deaths	p*	HR	95% CI
0/I and II	Negative	28	17 (60,7%)			
	Positiv0	27	20 (74,1%)	0,216	1,54	0,78 – 3,04
	Inconclusive	19	9 (47,4%)	0,676	1,20	0,52 – 2,76
III and IV	Negative	13	3 (23,1%)			
	Positive	27	13 (48,1%)	0,389	1,74	0,49 – 6,11
	Inconclusive	8	6 (75,0%)	0,015	5,66	1,39 – 23,1

*Cox regression model and Wald test, $p < 0.05$

Evaluation of agreement between ALCAM and ALDH1 and multivariate

The agreement between them was weak, with 22.1% agreement in both positive (n = 27) and 22.1% (n = 27) in both negative, out of a total of 122 patients evaluated. In the multivariate analysis of lung metastasis, clinical stage, and ALCAM marker, statistical significance was observed in the presence of lung metastasis. In contrast, the multivariate analysis between lung metastasis, clinical stage, and ALDH1 marker did not reveal statistical significance.

Multivariate analysis without the presence of markers

Multivariate analysis of the factors age, disease progression, primary tumor topography, post-surgical resection status, clinical stage, and presence of metastasis showed that there was statistical significance in age over 65 years, presence of disease progression, and incomplete surgical resection or residual tumor. When performing multivariate analysis of the factors age, disease progression, post-surgical resection status, clinical stage, and ALCAM positivity, statistical significance was found in age over 65 years, presence of disease progression, and incomplete surgical resection or residual tumor. According to multivariate analysis relating age, event progression, post-surgical resection status, clinical stage and ALDH positivity1, statistical significance was found in age over 65 years and presence of disease progression.

DISCUSSION

The age of patients at the time of colorectal cancer diagnosis is a very important factor in evaluating screening measures in health policies. Several classic studies show a low incidence below 50 years, with the median age at diagnosis being around 70 years in developed countries. However, in the present study, the mean age was 61.9 years. Regarding the distribution of patients by age group, the diagnosis was verified in 18% of the cases in the population under 50 years of age, a large portion in the 50-65 age group, which represents 36.9% of the cases, and in the group above, 45.1%. This data is important because it can represent a warning sign that colorectal cancer has a tendency to appear earlier and earlier. The demonstration of appearance under 50 years of age in a significant way may have an effect on screening programs, which today recommend complementary tests from the age of 50 in the general population.[6,7]

Although mutations in the BRCA gene have been described as being related to increased risk of colorectal cancer, with some studies hypothesizing that women in general would also have an increased risk, the results of this study showed a similar distribution between men and women, with a slight deviation to the male gender (51.6%). Population epidemiological studies show a similar distribution (49-52% males), which suggests the possibility of different causes according to gender, since if some factors increase the risk in females, others would increase them in males.[8-11]

Another piece of data that corroborates the idea that the etiological mechanism may be different between men and women is the significant difference in the location of the tumor. While and especially in older women, the most affected portion is the proximal colon, in men most involve the distal colon and rectum. The data presented here show a smaller distribution in the proximal colon (35.9%) than in the distal and

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rectum colons, which, when compared to other studies, present non-significant variations.[2,9,12,13]

Several studies have shown an association between more aggressive forms of the disease in the proximal colon, and a worse prognosis in women, since they are more prone to this location. Among the factors that can justify this difference in behavior is the possible difference between the etiological mechanisms and factors related to the diagnosis itself, such as the greater chance of false negative colonoscopic results in proximal colon tumors. However, the result of the present study did not show a significant difference in disease progression, both in terms of location and gender. On the contrary, the data were closer to a higher risk of in men, but not statistically significantly (SHR 1.63; CI 0.87-3.04)[4,8,12]

In this analysis, disease progression was characterized as new evidence of disease after the first treatment, either as local recurrence, worsening in NT staging, or even the appearance of metastases. In this sample, 35.2% of the patients had some type of tumor recurrence within 5 years, and it was not possible to observe incidence peaks, i.e., the recurrence had a linear distribution. Data on recurrence vary in the literature between 23-45% among patients undergoing resection with curative intent. This number raises several hypotheses both about the pathophysiology of recurrence, factors that may be associated with an increase or decrease in this risk, and, also, regarding postoperative follow-up.[14,15]

Regarding the factors that could influence the progression of the disease, no statistically significant results were obtained in relation to the location of the tumor, degree of tumor differentiation, clinical staging, presence or absence of distant metastases, or level of tumor resection (R0, R1, and R2). A significant association was found in relation to the presence of lung metastasis (SHR 4.33; CI 1.27-14.7). In addition, an important correlation between disease progression and the primary treatment modality was observed (SHR 3.30; CI 1.88-5.79); however, this variable presents a bias because it is dependent on clinical staging.

Wilhelmsen et al.[15] showed that numerous factors may be related to recurrence, such as mutations in the K-ras gene, microsatellite instabilities, among others. His study proposes subdivisions within clinical staging, based on treatment response, to better define the risk of recurrence in these specific patients. Some authors have studied associations that may reduce the risk of disease progression, such as statin use after the first treatment, but the results did not show an impact on progression itself, although they have an effect on overall survival.[14-18]

Regarding the markers analyzed, it was not possible to affirm that there is a prognostic relationship in their expression. Regarding ALCAM (CD166), the results showed that the minority of tumors were expressed differently from those found in the literature. However, the quantitative expression and the detailed cell site were not specifically characterized.[19-21]

Most studies that evaluated the association of ALCM with prognosis did not present data related to disease progression. These results showed that there was no significant correlation between ALCAM expression and recurrence (SHR 0.84; CI 0.43 – 1.65). Lugli et al.[19] obtained similar results, where there was no statistical significance in the correlation with local recurrence or metastases.[19]

ALDH1 expression is found in 70-80% of colorectal cancers, according to previous studies. However, in this sample, positive ALDH1 was found in 44.3% of 122 cases. A possible explanation for this difference was the high number of inconclusive samples for ALDH1 (11%, 27 cases), i.e., the technical difficulty in obtaining this result may have underestimated the number of cases with positivity for ALDH1.[20-23]

The relationship between ALDH1 expression and disease recurrence presents some results that are not statistically significant and others show an increased risk relationship that, when grouped in a meta-analysis, correlate ALDH1 with a worse 5-year disease-free survival rate, i.e., more recurrence events. Our results did not show statistical significance in this association, but suggest that, if grouped with other studies, they may reinforce the correlation of ALDH1 with greater disease progression (SHR 1.64; CI 0.79-3.40).[24]

Although few factors have shown a statistically significant correlation with events of disease progression, deaths have shown more associations. These results suggest a greater impact of other age-related factors (e.g., comorbidities) on the survival of this group of patients than disease progression.

When analyzing the entire sample, the factors with the greatest impact on the risk of death in 5 years were: degree of tumor differentiation with poorly differentiated neoplasm (HR 17.6; CI 3.5-88.6), tumor location in the rectum (HR 2.25; CI 1.13-4.48); disease progression in the form or local recurrence was the factor with the greatest impact on the risk of death at 5 years (HR 5.91; CI 3.37-10.4). In addition to these variables, the presence of liver metastasis was also related to death (HR 2.02; 1.17-3.47). The correlation between increased age and shorter survival agrees with several recent studies; however, there is still controversy about the probable cause of this correlation, whether they are associated comorbidities, degree of tumor differentiation, or isolated risk factor.[25,26]

Several studies have explored the potential differences in survival between tumors located on the right and left sides. In this sample, tumors located in the rectum had a worse prognosis in relation to survival when compared to other sites (HR 2.25; CI 1.13-4.48).

Benedix et al.[27] stated that, although distant metastases are more common in tumors on the left, survival is significantly worse on the right side, when grouped by clinical staging. Other studies show similar results, but contrast with a recent publication that, using multicenter data, showed no statistically significant difference between the two groups, with the exception of staging 3.[27-30] In addition, it is possible that the differences found are due to other variables possibly

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dependent on the location of the tumor, such as degree of differentiation and immunological differences, since the results show an association only in univariate analyses, and no publications with multivariate analyses were found.[12]

In agreement with the literature, the clinical staging at diagnosis was an important risk factor for death at 5 years, with stage III and IV patients having a 2.52-fold higher risk (CI 1.49-4.25). This data reinforces the importance of early diagnosis; however, even with the current screening programs, more than half (60.4%) of the patients were diagnosed in the most advanced stages (III and IV). This data is similar to that of emerging countries, as shown, for example, by a study in Malaysia where 58.6% were also in the late stages at the time of diagnosis.[25]

Developed regions, despite having the highest incidences of colorectal cancer, also have the highest 5-year survival rates. Early diagnosis has a great influence on this statistic, as only approximately 25% are diagnosed in the most advanced stages.[4,31]

In this study, ALCAM expression (CD166) did not show a statistically significant correlation with overall survival, which is in agreement with what was found by Lugli et al.[19], which used the largest sample found in the literature. Although this meta-analysis demonstrated the association of ALCAM with a worse prognosis (HR 1.94; CI 1.05-3.58), the existence of contradictory and borderline studies weighs more for the absence of CD166 impact than for its use as an independent prognostic marker.[3,19,20,21]

A possible explanation for the conflicting results is the lack of standardization in the ALCAM investigation in colorectal cancer, although the first study that investigated the prognostic association had already observed differences in this expression in different sites of the same cell, which may mask the results obtained in a less specific way. Another aspect presented by ALCAM is a nonlinear relationship with the degree of cell differentiation, which may indicate the need to apply some correction factor in its expression according to the histological classification to clarify the evolutionary pattern. However, the results presented here showed the same expression pattern if stratified according to clinical staging. When compared to stages I and II, it presented HR 0.36 (CI 0.13 – 1.03) and HR 1.43 (0.74 – 2.79) in stages III and IV.[4,32-34]

The association of ALCAM with other types of cancer and the results that, although controversial, show a relationship with prognosis in patients with colorectal cancer maintain this protein as a potential biomarker with clinical relevance; however, the biases are still too large to establish it as a strong prognostic marker.[21,35,36]

The results also showed no significant association between ALDH1 and overall patient survival (HR 1.09; CI 0.62 – 1.90). Previous studies have shown it as a strong marker of death within 5 years, but its expression presents subgroups with specific characteristics and, as with ALCAM, the results may be contaminated by the lack of specificity in the research technique.[20,24]

The refinement of ALDH1 for better analysis in relation to survival should be performed quantitatively and qualitatively, separating it into subgroups for a more specific identification of its role as a tumor marker. Fitzgerald et al.[37] showed that even among cases with positive ALDH1 expression, there is a significant difference between high and low expression. Other authors have stated that ALDH1 should be divided into subgroups according to their specific site of cell expression. The exact mechanism of its action in the pathogenesis of colorectal cancer is not known, but several studies have correlated its expression with the degree of local invasion and lymph node metastases.[21,24,33,37-39]

Future perspective

Although initial studies have pointed to ALCAM and ALDH1 as potential prognostic markers in colorectal cancer, there are still several points of conflict between the studies. These points are: 1) the event of disease progression in isolation, cases of lung metastasis, and those that were not treated surgically have a worse outcome; 2) death, also evaluated in isolation, is more present in patients with primary tumors of the rectum, advanced clinical stage (characterized by lymph node and metastatic disease), compromised surgical margins, presence of liver metastasis, and tumors with poorly differentiated histological grade; 3) lung metastasis, clinical stage, and ALCAM marker were statistically significant, which did not occur with ALDH1. To minimize these points, it is necessary to make the maximum refinement in the research of these substances, quantitatively and qualitatively, so that the results can be conclusive.

CONCLUSIONS

The immunohistochemical expression of the markers ALCAM and ALDH1 was not associated with the epidemiological and clinicopathologic characteristics evaluated. Regarding disease progression and death, it was also not possible to observe a relationship of correspondence with the markers evaluated.

Authors' contributions

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