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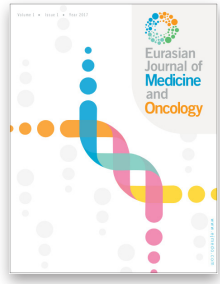

Research Article

VEGF and Ki-67 Expression in Colorectal Cancer: The Long-Term Impact on Recurrence and Mortality

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Abstract

Objectives: Colorectal cancer is the most frequent and mortal cancer in Portugal. Both angiogenesis and cellular proliferation are core mechanisms to tumoral progression, with VEGF (Vascular Endothelial Growth Factor) and Ki-67, respectively, being widely known markers of those two processes. The purposes of this study are to comprehend VEGF and Ki-67's impact on colorectal cancer prognosis which include assessing its expression in primary colorectal cancer of patients who underwent surgery, establishing associations between the expression of VEGF and Ki-67 and discovering hypothetical associations between these biomarkers and clinicopathological aspects, relapse, and mortality of patients.

Methods: A retrospective study was conducted in our hospital by including 512 patients submitted to surgery, from 2005 to 2010, with a post-operatory diagnosis of colorectal adenocarcinoma. The evaluation of expression of VEGF and Ki-67 in the obtained tissue was made through immunohistochemistry technique. The statistical analysis resourced to association tests and survival analysis.

Results: VEGF-A showed association with the variable gender (p-value of 0.016), with its expression being more frequent in men. VEGF-C expression is more common in colon than in rectum (p- value of 0.042). VEGF-C is significantly associated with Ki-67 (p-value of 0.036), with 69.7% of cases where both are positive. All markers are significantly associated with the grade of differentiation, with the VEGF family generally more present in well or moderately differentiated tumours and Ki-67 in the poorly differentiated. While the survival time was generally lower in the presence of any marker or combination, no significant differences were found among the survival analysis.

Conclusion: VEGF-A, VEGF-C and Ki-67 expression did not show impact on the prognosis of this sample of patients. There was no significant association with a poorer overall survival or a reduced disease-free survival.

Keywords: Colorectal cancer, Ki-67, mortality, recurrence, VEGF

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Colorectal cancer (CRC) is, according to the most recent available data, the third most diagnosed cancer in the world (10.0 %) and the second most mortal one (9.4%), only surpassed by lung cancer (18.0%).[1] In Portugal, since 2018, it has become the most frequent and mortal cancer, with approximately 10000 diagnosis and a mortality rate of about 45%.[2]

There are non-modifiable risk factors for the development

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of CRC, such as a personal or familial history of colorectal cancer and hereditary syndromes.[3] Age is a relevant contributing factor, and it's much more likely to develop CRC after the age mark of 50 years.[3] The male sex is also associated with a higher incidence.[4] In what concerns modifiable factors, dietetic habits, such as the excessive red meat consumption, a sedentary lifestyle, excessive alcohol consumption and smoking are identified behaviours that promote the incidence of this disease.[3,5]

CRC is a heterogenous group of cancers. It has been shown that the tumour location matters, with left-sided CRC being more common that right-sided CRC and rectum cancer.[4,6] Left- sided CRC's presentation is usually more evident, with rectal bleeding and bowel habits changes, as opposed to more gradual manifestations and consequently diagnosis at an advanced stage in right-sided CRC.[6]

Histologically, there are also different types of CRC, but adenocarcinoma is the predominant one.[7,8] Mucinous carcinomas, characterized by an over 50% content of extracellular mucins in tumour areas, represent 10% of CRC; Signet ring cell carcinoma, accounting for less than 1% of CRC, needs to have over 50% of signet ring cells to be classified as such.[8,9] Less frequent types of CRC include medullary carcinoma, adenosquamous carcinoma, among others.[9]

The the American Joint Committee on Cancer TNM system provides a stratification of cancer based on the invasion of the tumour, in this case, the invasion of the intestinal wall (T), involvement of lymph nodes (N) and the presence of metastasis (M). Four stages of CRC arise from this classification, with the 5-year survival rate decreasing over the latter stages. The treatment options that are available and effective, such as surgery (including metastectomy), perioperative chemotherapy and radiotherapy, takes into account the patient and the disease's extension.[9] In metastatic disease, anti-angiogenic agents, namely bevacizumab, are particularly relevant, and the increase in therapeutic options has contributed to gains in the overall survival (OS) of affected patients.[5,10]

There are many mechanisms involved in tumoral progression, with angiogenesis playing a very important role. Angiogenesis promotes the growth and metastatic process of CRC.[11-13] Among the molecules that intervene in this neovascularization, the family of the vascular endothelial growth factor (VEGF) is one of the most important pro-angiogenic factors.[12] VEGF acts through the binding to cell surface receptors and promotes endothelial proliferation and angiogenesis.[13] In the case of CRC, some studies have shown that the expression of these biomarkers is associated to metastatic disease and a poorer prognosis.[14-16] There is, nonetheless, controversy with contradictory results in the literature related to the value of VEGF as an outcome marker of this cancer.[17]

Another core mechanism of carcinogenesis is cellular proliferation. Ki-67 is a protein expressed in all phases of the cellular cycle, except G0, being a great marker of the cellular division and proliferation.[18] Its levels are increased in many malign tumours, and they are correlated with metastasis and poorer prognosis.[19] In the concrete case of CRC, however, the results are controverse: the high expression of Ki-67 was associated to earlier stage diagnosed cancers and to the lack of lymph node involvement, and it was considered a good prognosis factor concerning survival;[20] In other studies, was demonstrated that the high expression of this biomarker was correlated with poorer survival and a shorter disease-free interval.[19,21]

The complexity of angiogenesis and the inconsistencies among the literature, did not allow yet for it to be determined a predictive marker that could be widely used in the clinical practice.[11] On the same note, cellular proliferation's impact is also controverse, with ki-67 being described both as a poorer and greater prognosis marker in colorectal cancer.[19]

The combination of VEGF and Ki-67, its correlation with clinical and pathological parameters, as was their separate impact on prognosis has been evaluated in other types of cancer, such as squamous cell carcinoma of the tongue, non-invasive urothelial bladder cancer, uterine leiomyosarcoma, paediatric neuroblastoma, to comprehend if the expression of both has a different impact.[22-24,26] For example, in Park et al., the overexpression of the two markers showed a significantly worse prognosis regarding the OS in cystic adenoid cancer.[25] Having in consideration the importance of angiogenesis and cellular proliferation in the tumoral progression of CRC, but also the controversial results in the literature, the aims of this study are to comprehend VEGF and Ki-67's impact on CRC prognosis which include assessing its expression in primary CRC of patients who underwent surgery, establishing associations between the expression of VEGF and Ki-67 and discovering hypothetical associations between these biomarkers and clinicopathological aspects, relapse, and mortality of patients.

Methods

Study Design

An observational, analytic, retrospective, and descriptive study was conducted.

Participants

This project implied the collection of confidential data regarding the patients included in the sample. They were properly codified, and their anonymity is guaranteed.

After obtaining the approval of all the necessary institutions, we reviewed the charts of all patients submitted to surgery in Braga's Hospital during the time period of January 1st, 2005 to January 1st 2010, with the post-operatory histological diagnosis of colorectal adenocarcinoma. The exclusion criteria for the project were patients with a different histological diagnosis, who not submitted to surgical resection, who underwent palliative surgery or neoadjuvant chemo and/or radiotherapy, without enough information in the clinical process to determine the variables in study and in which it was not possible to recover the tissue piece of CRC. According to these criteria, a sample size of 512 patients was obtained.

Data Collection

Data was collected through access to the informatic clinical processes and registered in a Microsoft Excel confidential document. The following variables were assessed:

- Clinical and pre-operatory data: gender, age, oncologic personal history, familial history of CRC, clinical presentation, tumour location, pre-operatory value of carcinoembryonic antigen (CEA) and presence of metastasis on diagnosis;
- Histopathological data: size, macroscopic aspect, histological type, grade of differentiation, tumoral extension, lymph node involvement extension, lymphatic and blood vessels involvement, stage;
- Follow-up data: relapse and death;
- Biomarker's expression: VEGF and Ki-67.

Immunohistochemistry

The post-operatory tissue specimens of CRC were analyzed by immunohistochemistry for the expression of VEGF family markers, specifically VEGF-A and VEGF-C, and the Ki-67 protein. This analysis was previously conducted in other studies, having been thoroughly described then, and was performed by two independent pathologists to ensure reliability.[14, 27] For VEGF and Ki-67, the same method of evaluation of Immunohistochemistry was used. The extent of immunoreaction and the intensity of staining were recorded in a semi quantitative and semi qualitative manner, respectively, and the sum of both parameters created the final score.[14, 27]

The final score meaning was a little different among the two markers. In VEGF, 0 meant negative expression, 2 weak, 3 moderate and 4-6 strong. Only the moderate and strong were considered positive expression.[14] In Ki-67, 0-1 also meant negative, but a score of 2 or more meant a positive expression.[27]

Statistical Analysis

The statistical analysis was performed with IBM SPSS® Statistics, version 26.0. A p-value of less than 0.05 was considered statistically significant and the confidence interval was 95%.

A descriptive analysis was conducted with every variable. The association between the expression of the biomarkers VEGF-A, VEGF-C and Ki-67 and clinical and histopathological parameters was assessed with the Pearson Chi-Square test and Fisher's Exact Test if $n<5$. The correlation between the expression of VEGF markers and Ki-67 was evaluated through McNemar test. The OS and disease-free survival (DFS) curves were drawn using the Kaplan- Meier test for each protein marker and compared by the log-rank test. The predictive prognostic value of VEGF-A, VEGF-C and Ki-67 for survival and recurrence was analyzed by means of Cox proportional hazards regression, as was the combination of the markers who showed association.

Results

A sample of 512 patients was obtained and a descriptive analysis was performed. There were 319 patients of the male gender (62.3%) and 193 of the female gender (37.7%). Most of the individuals (488 patients) were over 45 years, accounting for 95.3% of the study population, while only 24 people were 45 or younger, a percentage of 4.7%.

When it comes to oncologic history, 499 individuals (97.5%) did not have any personal prior record and 430 people (84.0%) did not have familial history of cancer, with missing data in 40 cases.

At the time of diagnosis, 419 patients (81.8%) presented with clinical manifestations, while 93 (18.2%) were asymptomatic. Regarding the presence of metastasis, 406 patients (79.3%) did not present with metastasis while 106 patients (20.7%) did.

The tumour's location was the colon in 368 patients (71.9%), much more frequent than in rectum, with 144 cases (28.1%). The most frequent side of the colon affected was the left size, with 248 patients (71.9%) against only 120 individuals (23.4%) with right-sided colon cancer. Specifically, the most frequent location was the sigmoid colon, with 164 cases (34.3%).

The pre-operatory carcinoembryonic antigen value's cut-off was 10, with 83 patients (19.1%) having a higher value and 351 patients (80.9%) with a value of 10 or less; this information was not retrievable in 78 patients.

When it comes to the histological data, the tumour size was lower or equal to 4.5 cm in 295 patients (61.3%), and higher in 186 cases (38.7%). The macroscopic aspect was polypoid in over half of the patients (55.8%), followed by the ulcerative type (25.6%). The rarest was the villous, with only 2 cases, accounting for 0.4% of the population. The histological type was adenocarcinoma in 454 patients (88.7%), mucinous adenocarcinoma in 54 patients (10.5%) and adenocarcinoma with signet ring and mucinous cells were diagnosed in only 4 patients (0.8%).

At diagnosis, the most frequently found stage was 2, with 187 cases (36.5%), followed by stage 3, with 160 cases (31.3%), stage 1 with 89 individuals (17.4%) and, lastly, stage 4, with 76 people (14.8%). The grade of differentiation of tumours was more commonly well differentiated, in 225 patients, or moderately differentiated, with 213 cases (45,8% and 43,4%, respectively). Only 49 patients had a poorly differentiated cancer and 4 patients had undifferentiated CRC, which makes up for a percentage of 10.0% and 0.8%, respectively.

The extension of CRC to the lymph nodes was present in 210 individuals (57.7%), absent in 286 (42.3%) and unknown in 16 patients (3.1%). The lymph vessels involvement was present in 265 patients (55.7%) and absent in 211 (44.3%). The venous vessels invasion was less frequent, being present in 214 patients (43.9%) and negative in 273 (56.1%).

The immunohistochemistry comparison between normal and tumour epithelium can be seen in figures 1 and 2, regarding the expression of VEGF and ki-67, respectively.

The evaluation of the expression of VEGF biomarkers and Ki-67 was not possible in every patient. As demonstrated in Table 1, VEGF-A expression was the most found, in 98.1% of cases, followed by VEGF-C with a percentage of 92.3% and then Ki-67, in 68.4%.

As shown in Table 2, VEGF-C is significantly associated with Ki-67 (p-value: 0.036), with 69.7% of cases where they both are positive, 30.3% with VEGF-C positive and Ki-67 negative, 52.8% with Ki-67 positive and VEGF-C negative and 47.2% with both negative.

The association between VEGF-A, VEGF-C and Ki-67 and the clinical and histopathological parameters can be seen in table 3.

VEGF-A and the variable gender, for instance, with a p-value of 0.016, showed to be associated, with a more frequent positive expression of VEGF-A in men (99.3%) than in women (96.1%). VEGF-C and the variable tumour location, with a p-value of 0.042, showed that VEGF-C expression is more common in colon (93.8%) than in rectum (88.3%).

All markers showed a significant association with the variable grade of differentiation of CRC: VEGF-A with a p-value of 0.041, VEGF-C with a p-value of 0.043 and Ki-67 with a p-value of 0.036. VEGF-A was the most expressed in well differentiated tumours (99.1%), while still having a rele-

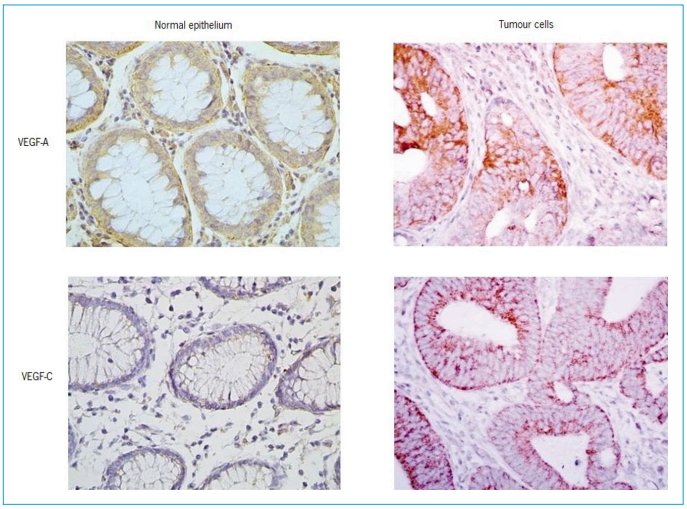

Figure 1. Immunohistochemical expression of VEGF family (original magnification x40).

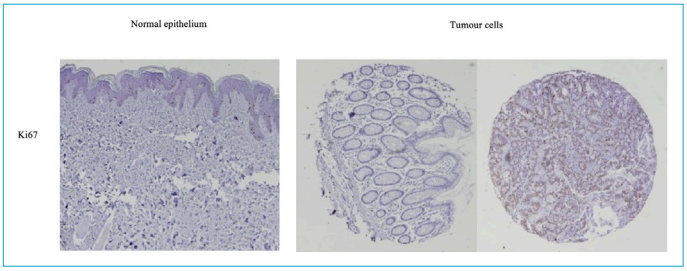

Figure 2. Immunohistochemical expression of Ki-67 protein (original magnification x40).

Table 1. Expression of the biomarkers

	Frequency (n)	Percentage (%)	Valid Percentage (%)
VEGF-A			
Positive	474	92.6	98.1
Negative	9	1.8	1.9
Total	483	94.3	100.0
Missing data	29	5.7	
VEGF-C			
Positive	453	88.5	92.3
Negative	38	7.4	7.7
Total	491	95.9	100.0
Missing data	21	4.1	
Ki-67			
Positive	333	65.0	68.4
Negative	154	30.1	31.6
Total	487	95.1	100.0
Missing data	25	4.9	

Table 2. Correlation between the markers

	VEGF-A				VEGF-C			
	Positive (%)	Negative (%)	Total	p	Positive (%)	Negative (%)	Total	p
Ki-67				.727				.036
Positive (%)	319 (68.6)	7 (77.8)	326		310 (69.7)	19 (52.8)	329	
Negative (%)	146 (31.4)	2 (22.2)	148		135 (30.3)	17 (47.2)	152	
Total	465	9	474		445	36	481	

vant expression in the moderate and poorly differentiated (97.5% and 97.9%, respectively). VEGF-C was more expressed in tumours with a moderate grade of differentiation (93.6%), followed by the well differentiated (93.1%) and accounting for 89.6% of the poorly differentiated. In contrast, Ki-67 was more expressed in tumours with lower grades of differentiation: its expression was the highest in poorly differentiated tumours (85.1%) and it was the most expressed in undifferentiated ones (75.0%).

The association between VEGF-C and Ki-67 and the aforementioned parameters was also evaluated. No significant differences were found for none of the clinicopathological variables.

The estimated mean of OS was approximately 115.0 months, and there were 237 deaths in a total of 408 cases (58.1%).

The survival curve of each marker is shown in figure 3 (a-c).

In the survival analysis of VEGF-A, the estimated mean of survival was approximately 113.6 months in total, with 114.2 months when there was no marker expression and 113.6 months in positive expression. The percentage of events was 58.7% when VEGF-A was expressed, but the log rank test showed there was no significance, with a p-value of 0.997.

In the survival analysis of VEGF-C, the estimated mean of survival was approximately 113.5 months in general, with 134.3 months in negative expression and 111.1 months in positive. There were 60.1% events when there was VEGF-C expression but without significance (p- value of 0.105).

When it comes to Ki-67, the estimated mean of survival was 115.4 months in general, with 119.1 months in negative and 113.6 in positive expression. Despite there were 59.2% events in positive expression, the p-value was 0.483.

There were no significant differences with regard to OS when evaluated with the expression of markers. The number of events was higher when each biomarker was expressed than in its absence, but no association was demonstrated between them.

In the combination of VEGF-C and Ki-67, markers who demonstrated a significant correlation with each other, the estimated survival mean was 115.2 months, with 123.6 months when this co-expression was absent and 110.0 months in its presence. The p-value was 0.093, which, although lower than those mentioned above, still shows no significant differences. The percentage of events when both markers were expressed was 61.4%.

When it comes to recurrence, this event took place in 113 cases in the total of 391 individuals, which represents a percentage of 28.9%. The estimated mean time to relapse was 135.7 months. The assessment of time to relapse in the presence of each marker is shown in figure 4 (a-c).

In the disease-free interval analysis of VEGF-A and VEGF-C, the estimated mean was approximately 134.1 months in general.

When VEGF-A or VEGF-C were expressed, both estimated mean time to recurrence was approximately 127.0 months. There were 29.3% of events in VEGF-A positive expression and 28.6% in case of VEGF-C.

When there was no marker expression, the estimated mean time of DFS was 149.3 months to VEGF-A and 131.6 months to VEGF-C.

When it comes to Ki-67, the estimated mean time of DFS was 134.0 months in general, with 123.0 months in negative and 137.6 in positive expression. There were 29.1% events in positive expression.

Similar to OS, there were no significant differences about the DFS when evaluated with the expression of markers: In VEGF-A, the p-value was 0.575; in VEGF-C, it was 0.796; in Ki-67, it was 0.768.

With the expression of VEGF-C and Ki-67, the estimated mean time of DFS was 135.3 months in general, 130.2 months when they were both expressed and 131.4 months when they weren't. There were 27.8% of events with this combination. This combination did not show a significant association with disease-free interval, with a p-value of 0.662.

Table 3. Assessment of association between VEGF-A, VEGF-C and Ki-67 and clinic and histopathological parameters

	VEGF-A			VEGF-C			Ki-67		
	Total	Positive (%)	p	Total	Positive (%)	p	Total	Positive (%)	p
Gender			.016*			.933			.675
Male	303	301 (99.3)		307	283 (92.2)		307	212 (69.1)	
Female	180	173 (96.1)		184	170 (92.4)		180	121 (67.2)	
Age			1.00			.684			.428
≤45 years	21	21 (100)		22	20 (90.9)		23	14 (60.9)	
>45 years	462	453 (98.1)		469	433 (92.3)		464	319 (68.8)	
CRC personal history			.219			1.00			1.00
Present	13	12 (92.3)		13	12 (92.3)		13	9 (69.2)	
Absent	470	462 (98.3)		478	441 (92.3)		474	324 (68.4)	
CRC familial history			1.00			.756			.833
Present	39	39 (100)		39	37 (94.9)		40	28 (70.0)	
Absent	405	397 (98.0)		412	379 (92.0)		408	279 (68.4)	
Presentation			.199			.575			.474
Symptomatic	398	392 (98.5)		404	374 (92.6)		399	270 (67.7)	
Asymptomatic	85	82 (96.5)		87	79 (90.8)		88	63 (71.6)	
CEA (ng/mL)			1.00			.982			.750
≤10	333	326 (97.9)		337	311 (92.3)		337	229 (68.0)	
>10	74	73 (98.6)		77	71 (92.2)		73	51 (69.9)	
Metastasis at diagnosis			.398			.965			.223
Present	99	96 (97.0)		102	94 (92.2)		98	62 (63.3)	
Absent	384	378 (98.4)		389	359 (92.3)		389	271 (69.7)	
Tumour location			.713			.042			.764
Colon	349	343 (98.3)		354	332 (93.8)		353	240 (68.0)	
Rectum	134	131 (97.8)		137	121 (88.3)		134	93 (69.4)	
Macroscopic appearance			.723*			.072*			.182*
Polypoid	249	244 (98.0)		254	229 (90.2)		253	171 (67.6)	
Ulcerative	114	111 (97.4)		114	109 (95.6)		111	76 (68.5)	
Infiltrative	37	36 (97.3)		39	36 (92.3)		38	22 (57.9)	
Exophytic	38	38 (100)		38	37 (97.4)		39	32 (82.1)	
Villous	2	2 (100)		2	1 (50.0)		2	2 (100)	
Tumour size (cm)			.162			.229			.519
≤4.5	280	273 (97.5)		281	263 (93.6)		279	190 (68.1)	
>4.5	174	173 (99.4)		180	163 (90.6)		179	127 (70.9)	
Histological type			1.00*			.695*			.538*
AdenoCa	430	422 (98.1)		436	403 (92.4)		433	299 (69.1)	
Mucinous AdenoCa	49	48 (98.0)		51	46 (90.2)		50	32 (64.0)	
Signet cell and mucinous AdenoCa	4	4 (100)		4	4 (100)		4	2 (50.0)	
TMN Stage			.719*			.373*			.711*
1	86	83 (96.5)		86	76 (88.4)		84	59 (70.2)	
2	182	179 (98.4)		180	170 (94.4)		181	121 (66.9)	
3	147	145 (98.6)		156	143 (91.7)		152	108 (71.1)	
4	68	67 (98.5)		69	64 (92.8)		70	45 (64.3)	
Grade of differentiation			.041*			.043*			.036*
Well	211	209 (99.1)		216	201 (93.1)		209	135 (64.6)	
Moderate	203	198 (97.5)		204	191 (93.6)		208	146 (70.2)	
Poor	48	47 (97.9)		48	43 (89.6)		47	40 (85.1)	
Undifferentiated	3	2 (66.7)		4	2 (50.0)		4	3 (75.0)	
Extension to lymph nodes			.742			.958			.940
Present	193	190 (98.4)		202	187 (92.6)		198	136 (68.7)	
Absent	275	269 (97.8)		274	254 (9.7)		275	188 (68.4)	
Lymph vessels invasion			1.00			.881			.765
Present	246	241 (98.0)		254	233 (91.7)		250	172 (68.8)	
Absent	204	200 (98.0)		203	187 (92.1)		204	143 (70.1)	
Venous vessels invasion			1.00			.086			.511
Present	195	191 (97.9)		202	191 (94.6)		201	142 (70.6)	
Absent	266	261 (98.1)		266	240 (90.2)		264	179 (67.8)	

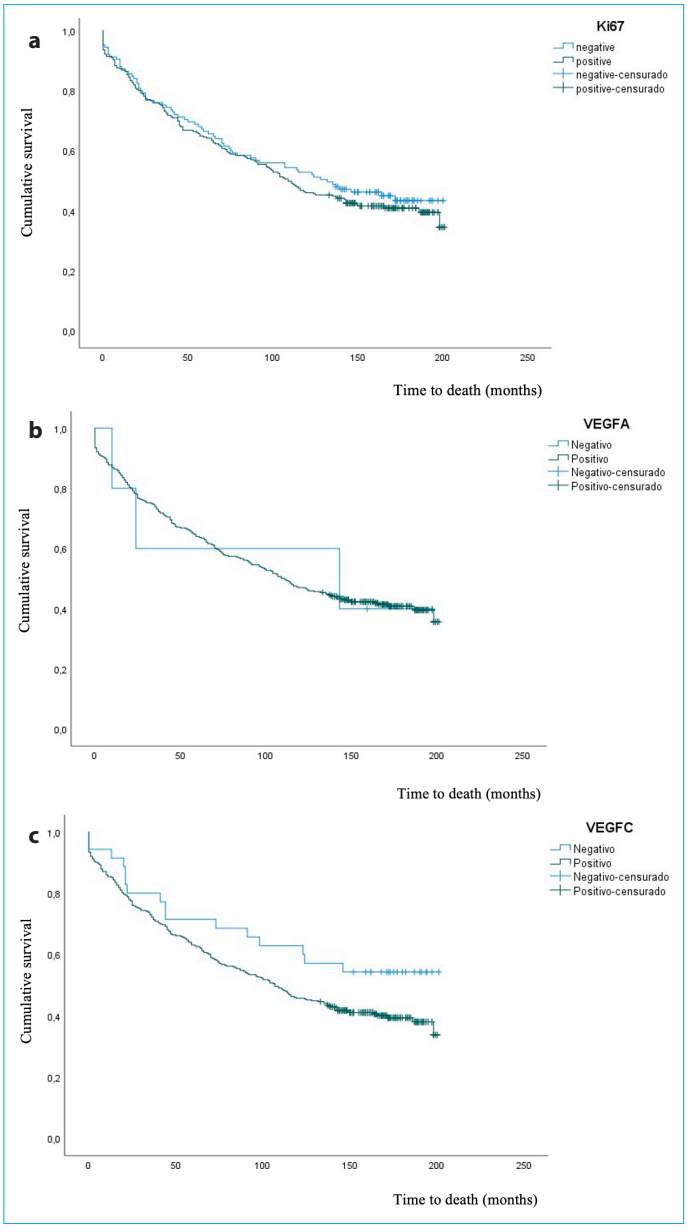

Figure 3. OS, regarding the follow up until October 31st, 2021: **(a)** VEGF-A; **(b)** VEGF-C; **(c)** Ki-67.

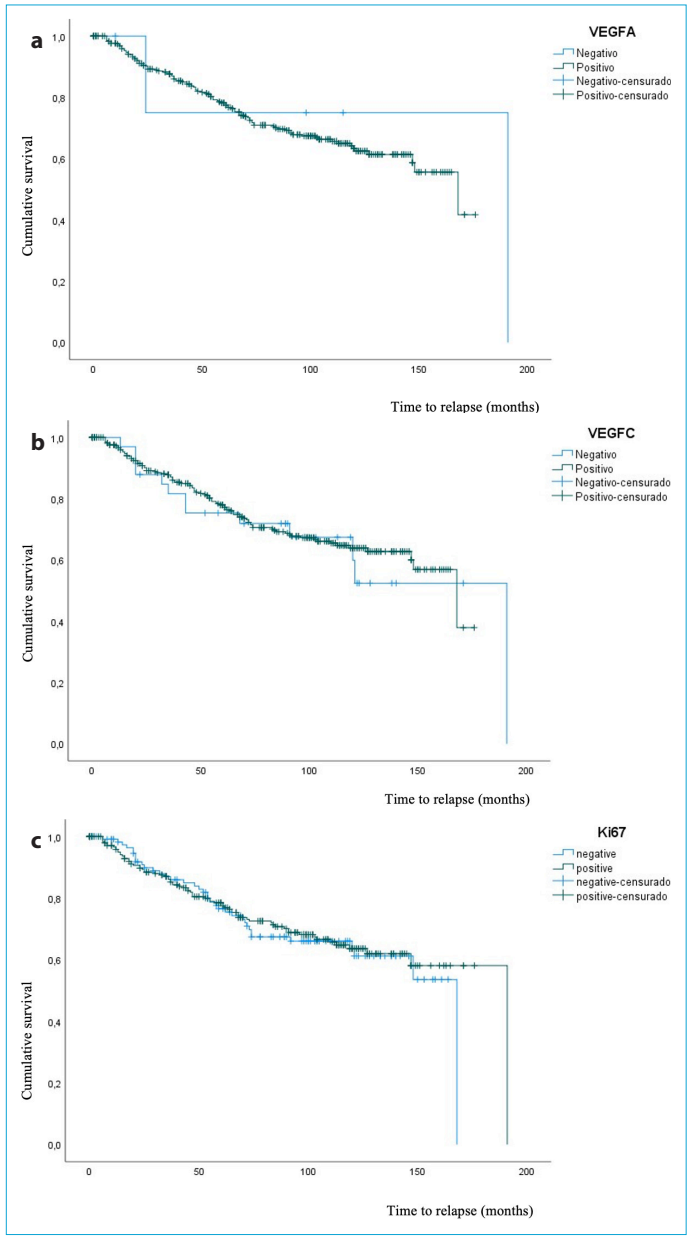

Figure 4. DFS, regarding the follow up until October 31st, 2021: **(a)** VEGF-A; **(b)** VEGF-C; **(c)** Ki-67.

Discussion

In our sample, most individuals were of the male gender and with an age of 45 years or superior, which is concordant with how CRC behaves in general.[3,4] The predominant histological type was adenocarcinoma, as is in the literature.[7,8]

Both VEGF-A and VEGF-C expression was widely found (98.1% and 92.3%, respectively), and Ki-67 was expressed in 68.4% of the samples in which its determination was possible. According to the results, VEGF-A had a significant association with gender, being more frequently positive in men and VEGF-C showed association with the tumour location, being more commonly found in colon rather than rectum. Both markers from the VEGF family also had a significant association with the grade of differentiation of tumours, and had a prominent expression in well, moderate, and poorly differentiated tumours. VEGF-A was the most found in well, moderate, and poorly differentiated tumours, and its highest percentage was found in the well differentiated. VEGF-C followed a similar pattern, having its highest expression in the moderately differentiated, but still being frequently found in the well and poorly differentiated. The association of VEGF with the grade of differentiation of CRC is not consensual. Some studies have associated VEGF with tumours

with poor differentiation.[28] However, others showed an association between these markers and well differentiated tumours, and while in our study VEGF expression was still very relevant in poorly differentiated, its expression was higher in the well and moderately differentiated as well.[29]

VEGF-A is one of the most potent angiogenic factors, and its high expression in CRC has been found in other studies as well.[30] It has been associated with metastatic tumours and a generally poor prognosis, but our study did not find significant associations with metastasis, invasion, relapse, or OS.[14]

VEGF-C is mainly implicated in the process of lymphangiogenesis and while it has been associated with lymphatic node metastasis in some studies regarding other types of cancer, that is not always the case.[31] As demonstrated by George et al., in the case of colorectal cancer, no significant differences were found between VEGF-C mRNA expression and lymphatic involvement.[30] Our study corroborates the latter since no significant association was found between this marker and lymph nodes invasion.

The expression of the VEGF family in CRC tissue samples has been a target of many studies, as well as its prognostic utility, but the results haven't been clear.[14, 15, 17, 28, 29] Ferroni et al. concluded that VEGF had a negative prognostic impact in DFS and OS and, similarly, Zafirelis et al. identified VEGF as a predictor of negative outcomes regarding disease-specific survival.[32, 33] Zheng et al., Lee et al. and Khorana et al. all indicated that VEGF did not have prognostic value.[28, 34, 35] Our results are concordant with the latter studies since neither VEGF-A or VEGF-C showed a significant impact on DFS and OS.

On the case of Ki-67, we found a significant association with the grade of differentiation of tumours, with its expression being more relevant in the poorly differentiated tumours, and it was the most found in undifferentiated ones. This finding agrees with the literature, which showed its expression can be higher in less differentiated tissues.[19, 36, 37]

Regarding the impact of Ki-67 on prognosis, the reported findings in the literature cause controversy. In some studies, high Ki-67 expression indicates a favourable prognosis.[20, 38] On another hand, Tong et al. showed that a high expression of Ki-67 was associated with a more invasive tumour and was found to be an independent predictor of poor prognosis in CRC.[19] A similar result was found in Luo et al., a meta-analysis that concluded that high expression of Ki-67 was significantly associated with a worse prognosis as well.[21] Jansson et al. did not find any association between Ki-67 and survival, which concurs with our own findings: no significant correlation was found between this marker and OS or DFS, thus showing no considerable impact on prognosis.[39]

The VEGF and Ki-67 relationship in CRC was previously found to be significant in Van Triest et al., and our study corroborated that result, finding an association between VEGF-C and Ki-67.[40] The combination of VEGF and Ki-67 has been studied in other types of cancer, to comprehend if the expression of both has a different impact. In Park et al., the overexpression of the two markers showed a significantly worse prognosis regarding the OS in cystic adenoid cancer.[25] Chen et al. created a molecular model combining Ki-67 and VEGF considering the thresholds of each marker that provided a good separate model of recurrence prediction. Then the utility of the combination was demonstrated, with an increase of sensitivity and specificity in this evaluation.[26]

In the present study, we did not find a significant impact on the OS or DFS with this combination of markers. In the OS, however, a tendency to a worse survival was seen, even though no significant differences were found (p-value: 0.093).

The discrepancies previously described between the results found among the various studies have many sources. The sample size, non-modifiable characteristics of the population, immunohistochemistry specificities, the scoring method for immunohistochemistry, are some examples of limitations that have been pointed out in these studies that can explain these differences.[33, 37] The heterogeneity of CRC itself has also been considered a contributing factor.[37]

The absence of a more concise definition of the thresholds of interest of each marker, as made in Chen et al.[26] can be appointed as a limitation to our results. The standardisation of significant predictive cut-offs of the markers for the desired outcomes, such as OS and DFS, could produce more definitive results. The lack of follow-up data, which led to unretrievable information in many categories and the exclusion of patients is also a limitation, but a necessity to guarantee realistic results.

Conclusion

In conclusion, the expression of the markers involved in this study (VEGF-A, VEGF-C, and Ki-67) did not show a significant impact on the studied outcomes: OS and DFS. The combination of markers who had a significant association between one another (VEGF-C and ki-67) also showed no association with the prognosis of these patients.

Given the relevant prevalence of CRC and its mortality, it is imperative to continue this type of research, in order to better understand which targeted therapies could be more useful to use and when, and which novel targets should be. Since these markers still have discrepant findings in the literature, it is important to clarify them further.

Disclosures

Ethics Committee Approval: The study got the approval of all the necessary institutions: Ethics Commission for Sciences of Life and Health of Minho's University, Data Protection Officer of Braga's Hospital and the Ethics Commission for Health of Braga's Hospital.

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