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Evaluation of tumor-infiltrating lymphocytes and molecular alterations in advanced colorectal carcinoma: a retrospective study in Southern Brazil

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Abstract

Background Colorectal carcinoma (CRC) is a leading cause of cancer-related deaths globally, ranking second in mortality rates. Cancer progression is influenced not only by genetic mutations in tumour cells but also by the surrounding tumour microenvironment, which can promote tumour growth or dampen the host's immune response. Microsatellite instability tumours often exhibit a high presence of tumour-infiltrating lymphocytes (TILs), including cytotoxic T cells. TILs are increasingly recognised as important biomarkers across various cancer types, and the mismatch repair (MMR) status is particularly relevant in determining patient eligibility for immunotherapy, especially with immune checkpoint inhibitors in advanced disease. In the present study, we evaluated the presence and intensity of lymphocytic infiltrate in patients with advanced CRC at a tertiary hospital in southern Brazil.

Methods A cross-sectional retrospective study was conducted to analyse the presence and intensity of TILs and their association with clinical data, as well as alterations in *KRAS*, *NRAS*, *BRAF*, *NTRK*, and MMR.

Results Analysis of the presence and intensity of TILs in 241 tumours revealed that 70 (29.1%) were TIL + and 171 (70.9%) were TIL−. Only one tumour was *NTRK*+; this tumour was in a female patient, located in the right colon, TIL−, and deficient in MLH1/PMS2. There were no significant differences in the associations between the presence and intensity of the infiltrate and the clinical characteristics and molecular alterations studied.

Conclusions The data from our study differed from those reported in the literature in that we found no association between a higher frequency and intensity of TILs and MMR deficiency. Associations with the molecular profiles of the *KRAS*, *NRAS*, and *BRAF* genes also showed no statistically significant differences.

Keywords Colorectal carcinoma, TILs, MMR, Molecular status

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Introduction

Colorectal carcinoma (CRC) is one of the most commonly diagnosed cancers worldwide, ranking second in cancer mortality [1]. Cancer progression is influenced not only by genetic alterations within tumour cells but also by the surrounding environment, which can provide factors that either promote cancer growth or reduce the host's immune surveillance [2]. The microsatellite instability (MSI) pathway is recognised as a carcinogenic pathway in CRC and is found in 15% of sporadic colorectal tumours [3, 4]. MSI is a phenotype caused by mutations or epigenetic silencing of the mismatch repair (MMR) system genes. Because the MMR system's repair proteins correct errors during DNA replication, MSI tumours with MMR deficiency (dMMR) exhibit a high mutational tumour burden and a high number of neoantigens, which are recognised by the immune system [5]. These tumours are generally characterised by an increased number of tumour-infiltrating lymphocytes (TILs), including cytotoxic T cells [6–8]. By contrast, MMR-proficient colorectal tumour cells have low immunogenicity and are infiltrated by a limited number of immune cells, making it difficult to elicit an adequate immune response [9]. Therefore, immune checkpoint blockade (ICB) therapy is ineffective in such patients. To enhance the sensitivity of immunotherapy, combination treatments are needed to boost tumour immunogenicity. The presence of TILs is increasingly recognised as an important biomarker in multiple cancer types [10], and the MMR status is gaining attention as a biomarker for determining patients' eligibility for immunotherapy with immune checkpoint inhibitors, especially for those with advanced disease [7]. Generalised TIL density is a strong prognostic marker for survival in patients with colorectal cancer [10]. ICB therapy works by inhibiting interactions between molecules such as cytotoxic T-lymphocyte associated protein 4 and programmed death-1 (PD-1), which normally suppress T cell activation and function [2]. Tumours with a high mutational load and therefore increased immune infiltration respond favourably to ICB [11]. ICB has provided certain patients with long-lasting benefits and a significantly improved disease prognosis. However, inhibition of PD-1 or programmed cell death-ligand 1 (PD-L1) therapy has shown limited effects in the treatment of colorectal cancer.In 2017, the first anti-PD-1 drug, pembrolizumab, was approved by the Food and Drug Administration as a second-line treatment for patients with metastatic CRC who have MSI-high (MSI-H) tumours. However, only a small proportion of patients have dMMR/MSI-H tumours (approximately 15% of patients with colorectal cancer and 4% of those with metastatic CRC), and some of these patients develop immune resistance. In this study of patients with advanced CRC at a tertiary hospital in southern Brazil, we evaluated the presence and intensity of intratumoural lymphocytic infiltrate (i.e., TILs) in relation to the mutational status of *KRAS*, *NRAS*, and *BRAF*. We also examined protein alterations detected by immunohistochemistry of the *NTRK* and MMR status and histopathological features of advanced CRC.

Methods

Study population and sample

This retrospective study utilised data from a series of patients with clinical stage III or IV CRC treated at the Hospital de Clínicas de Porto Alegre. Samples were obtained from the Surgical Pathology Service between 2018 and 2022, with patient consent for their use. Primary site tumour samples were collected from surgical specimens and biopsies, and 241 patients were included in this study. Clinical and pathological data were obtained through a review of original patient medical records. The study was approved by the Research Ethics Committee of the Hospital de Clínicas de Porto Alegre under CAAE number 56.230.122.200.005.327.

Evaluation of TILs

The evaluation and semi-quantification of TILs were performed with the same haematoxylin–eosin slides used in the histopathological diagnosis. Briefly, formalin-fixed, paraffin-embedded samples were cut on a microtome set at 3 μm, and haematoxylin–eosin staining was carried out using automated equipment (VENTANA HE 600 system; Ventana Medical Systems Inc., Tucson, AZ, USA). TIL evaluation in the surgical sections was conducted in two regions: the centre of the lesion and the margin. For biopsies, the tumour infiltrate at its specific location was considered for analysis. Microscopic evaluation was performed initially at 100x magnification, increasing to 400x, and the results were reported in four intensity levels: absent, weak (rare lymphocytes), moderate (focal infiltrate), and strong (diffuse infiltrate), according to Liu et al. [12]. After semi-quantification, the results were categorised as negative TILs (absent or weak lymphocyte infiltrate) or positive TILs (moderate or strong lymphocyte infiltrate).

V600E, *KRAS*, *NRAS* and MMR status

The patient tumour tissue specimens in this study were analysed for their molecular status, including the *BRAF* V600E mutation and *KRAS* and *NRAS* mutations (exons 2, 3, and 4), by next-generation sequencing. The MMR status was assessed by immunohistochemistry as previously described by Remonatto et al. [13].

Evaluation of NTRK expression

NTRK expression was analysed by immunohistochemistry using a Roche monoclonal anti-pan-TRK antibody,

clone EPR17341, which recognises proteins resulting from the fusion of *NTRK* genes. This was performed on an automated BenchMark ULTRA Ventana® platform, using material from the primary tumour site. For the surgical specimen samples, tissue microarray (TMA) blocks were created based on the anatomical examination of each patient's specimen. Each TMA block contained 60 tumour cylinders of 2 mm, with duplicate tumours for each patient. For the biopsy blocks, a TMA was not constructed, and a single slide was prepared for each patient. The results were reported as either positive or negative for *NTRK*.

Statistical analysis

Statistical analyses were conducted using SPSS for Windows, version 18 (IBM Corp., Armonk, NY, USA). To investigate the association of TILs with sex, age, tumour location, molecular status, MMR status, histological grading, and mucinous component, the $\chi 2$ test or Fisher's exact test was performed. Results were considered statistically significant when $P < 0.05$.

Results

Clinicopathological characteristics of patients

The present study involved 241 patients (124 women and 117 men). The tumour was located in the right colon in 56 patients, the left colon in 115, and the rectum in 66. In four patients, the tumour location was not specified. The mean age at diagnosis was 59 years, with a median of 61 years (range: 18–84 years). Forty-one (17%) patients had tumours with a mucinous component, and 81.7% were moderately differentiated (Table 1).

Table 1 Clinicopathological characteristics of patients

Clinical data	*n*	%
Sex		
Male	117	48.5
Female	124	51.5
Site		
Right colon	56	23.2
Left colon	115	47.7
Rectum	66	27.4
Not specified	4	1.7
Age		
Under 30 years	7	2.9
31-40 years	9	3.7
41-50 years	38	15.8
51-60 years	60	24.9
61-70 years	75	31.1
71-80 years	48	19.9
Over 80 years	4	1.7
Histological grading		
Undifferentiated	1	0.4
Poorly differentiated	26	10.8
Moderately differentiated	197	81.7
Moderately to poorly differentiated	1	0.4
Well differentiated	11	4.6
Not informed	5	2.1
Mucinous component		
Positive	41	17.1
Negative	200	82.9

Evaluation of TILs

When assessing the intensity of TILs, 54 (22.4%) samples showed no infiltrate, 117 (48.5%) showed a weak infiltrate, 62 (25.7%) showed a moderate infiltrate, and 8 (3.3%) showed a strong infiltrate (Figs. 1 and 2). In the stratification between TIL + and TIL–, 70 (29.1%) tumours were TIL + and 171 (70.9%) were TIL–

Relationship between TILs and clinicopathological characteristics

The evaluation of TILs and patient characteristics did not reveal a statistically significant difference in any of the variables analysed (Table 2). The primary site with the highest percentage of TIL + was the rectum, with 34.9%. Patients under the age of 30 years had 42.9% of TIL + tumours (3 of 7 tumours).

Relationship between TILs and molecular status

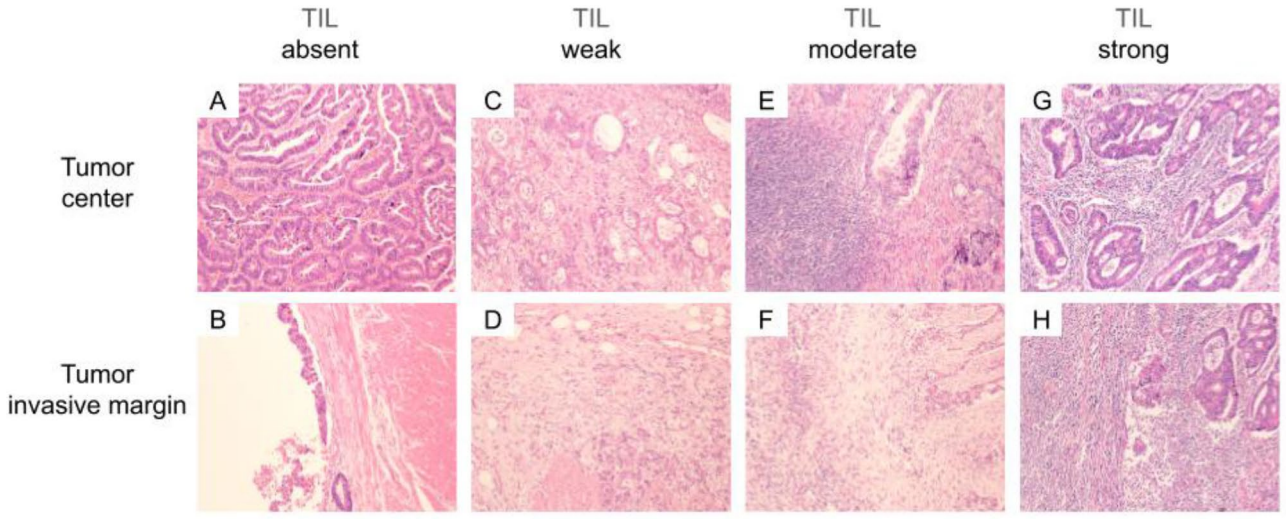
Of the 241 patients included in this study, 239 were tested for MMR and 235 for *NTRK*. The remaining patients did not have a viable sample. The results are presented in Table 3. When analysing TILs and the molecular status, no statistically significant differences were observed.

Regarding the association between TILs and MMR, most tumours deficient in MLH1/PMS2 were TIL–, while the majority of tumours deficient in MSH2/MSH6 were TIL+ (Fig. 3).

Discussion

This study evaluated the presence and intensity of TILs and associated the findings with clinicopathological characteristics and mutational profiles in patients with advanced CRC at a hospital in southern Brazil.

Specific features of the tumour microenvironment, including TILs, the Immunoscore, and PD-L1 expression, may predict responses to ICB [14].Research is increasingly highlighting the importance of tumour immune infiltration, which involves various immune cells such as T cells, B cells, natural killer cells, macrophages, dendritic cells, and neutrophils, and underscoring significant variability between patients [15, 16]. The first description in CRC of the prognostic value of tumour-infiltrating T cells, specifically the density and location of CD8 + cytotoxic T cells and Th1 cells, was reported by Galon et al. in 2006 [17]. Based on these findings, the concept of the Immunoscore as a more effective prognostic factor than pathological tumour progression (T-stage), tumour



Levels of intensity A and B absent, C and D weak (rare lymphocytes), E and F moderate (focal infiltrate), G and H strong (diffuse infiltrate). Magnification of 100x.

Fig. 1 Assessing of TIL intensity in th center and at the invasive margin of the tumor

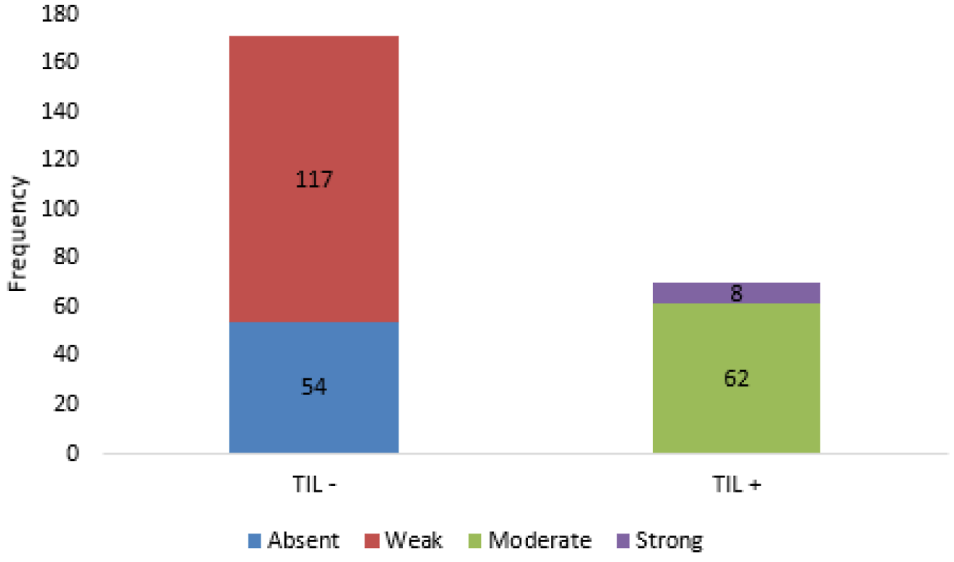


Fig. 2 Frequency of tumor lymphocyte infiltration by intensity TIL: Tumor-infiltrating lymphocyte

invasion (N-stage), tumour metastasis (M-stage), TNM staging, and the MSI status was proposed [18]. Colorectal cancer has long been considered immunogenic and challenging to treat with immunotherapy [15, 16]. However, advancements in the molecular characterisation of tumour-associated antigens and methods for detecting antigen-specific T-cell responses have shifted this perspective within the scientific community. The presence of TILs in tumours has been associated with improved clinical outcomes. In 2018, Mlecnik et al. demonstrated that in the metastatic context, response to treatment and prolonged survival were significantly associated with high immune infiltration [19].However, the type and function of TILs, as well as the localisation of different TILs within the tumour microenvironment, are crucial for determining whether tumour control or progression occurs [20]. In our study, when evaluating the frequency and intensity of TILs, we observed that of the 241 tumours tested, 70 showed moderate to strong infiltration, characterising TIL + samples according to Liu et al. [12].

Literature data associate the presence and intensity of TILs with a deficiency in the MMR system due to the increased expression of neoantigens resulting from uncorrected DNA replication errors [8, 15]. In our study,

Table 2 Relationship between TIL and clinicopathological characteristics

Clinical data	TIL					
	Negative			Positive		
	Total	n	%	n	%	*P*
Sex						
Male	117	85	72.6	32	27.4	0.674
Female	124	86	69.4	38	30.6	
Site						
Right colon	56	40	71.4	16	28.6	0.649
Left colon	115	85	73.9	30	26.1	
Rectum	66	43	65.1	23	34.9	
Not specified	4	3	75	1	25	
Age at diagnosis						
Under 30 years	7	4	57.1	3	42.9	0.712
31-40 years	9	7	77.8	2	22.2	
41-50 years	38	25	65.8	13	34.2	
51-60 years	60	41	68.3	19	31.7	
61-70 years	75	57	76	18	24	
71-80 years	48	35	72.9	13	27.1	
Over 80 years	4	2	50	2	50	
Histological grading						
Undifferentiated	1	1	100	0	0	0.513
Little differentiation	26	21	80.8	5	19.2	
Moderately differentiated	197	138	70.1	59	29.9	
Moderately to poorly differentiated	1	0	0	1	100	
Well differentiated	11	8	72.7	3	27.3	
Not informed	5	3	60	2	40	
Mucinous component						
Negative	200	139	69,5	61	30,5	0.363
Positive	41	32	78.1	9	21.9	

TIL: Tumor infiltrating lymphocytes

Table 3 Relationship between TIL and molecular status

Molecular status	TIL					
	Negative			Positive		
	Total	n	%	n	%	*P*
KRAS						
Wild	119	84	70.6	35	29.4	1
Mutated	122	87	71.3	35	28.7	
NRAS						
Wild	232	166	71.5	66	28.5	0.246
Mutated	9	5	55.5	4	44.5	
BRAF						
Wild	219	155	70.8	64	29.2	1
Mutated	22	16	72.7	6	27.3	
MMR						
Deficient	23	17	73.9	6	26.1	0.909
Proficient	216	152	70.4	64	29.6	
NTRK						
Negative	234	164	70.1	70	29.9	0.702
Positive	1	1	100	0	0	

TIL: Tumor infiltrating lymphocytes

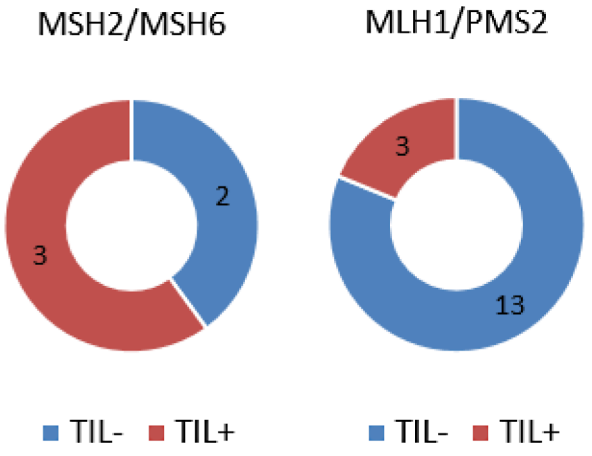


Fig. 3 Frequency of tumor lymphocyte infiltration by type of MMR deficiency.TIL: Tumor infiltrating lymphocytes

we found that patients with MSH2/MSH6 deficiency had more TIL + than TIL – tumours, unlike what was observed in tumours deficient in MLH1/PMS2. This finding could suggest that MSH2/MSH6 dMMR tumours may attract more TILs, although this association was not statistically significant. We also found no statistically significant association between TILs and MMR overall. This may have been due to the small number of dMMR patients evaluated, likely because only patients at an advanced clinical stage were included in the study [21].

When we analysed the association between TILs and *KRAS* mutations, we found that the proportion of TIL + was the same in mutated *KRAS* as in wild-type *KRAS*. This finding is consistent with the literature [22]. In the analysis of TILs and the *NRAS* gene, we observed that of the 9 patients with mutated *NRAS*, 4 had TIL + and 5 had TIL–. Because our previous study showed an association between dMMR and the *BRAF* V600E mutation, we expected some form of association with TILs as well. However, because we did not find any association between dMMR and TILs, the same result was observed when we analysed the *BRAF* gene.

With regard to the *NTRK* analysis, of the 235 patients analysed, only one was *NTRK*+ (0.42%). This is consistent with the very low prevalence of *NTRK* fusions in CRC (< 1%) [23, 24]. This sample belonged to a woman who was 66 years old at the time of her CRC diagnosis. The tumour was poorly differentiated, lacked a mucinous component, and was located in the right colon. It was dMMR (MLH1/PMS2), wild-type *KRAS*, wild-type *NRAS*, and wild-type *BRAF*, with a weak lymphocytic infiltrate (TIL–). This patient could be eligible for treatment with ICB drugs such as pembrolizumab and TRK inhibitors like entrectinib and larotrectinib [15, 25–27]. Literature data support the relationship between dMMR and *NTRK* fusions, consistent with our findings [28, 29].

The main limitations of this study are the low number of dMMR patients analysed and the lack of data on clinical treatment, prognosis, and patient survival. A methodological limitation was the absence of immunohistochemical characterisation of the lymphocytes that make up the infiltrate.

Although our findings did not reveal a statistical association between the variables analysed, the study has some strengths. These include the ability to associate TIL-related findings with the molecular status of *KRAS*, *NRAS*, and *BRAF*, as well as the immunohistochemical characterisation of MMR and *NTRK*.

Conclusions

This study analysed the frequency and intensity of TILs and investigated associations between these data and the molecular and immunohistochemical profile of advanced CRC. Our findings differed from those reported in the literature in that we found no association between a higher frequency and intensity of TILs and MMR deficiency, which may be attributed to the small number of dMMR patients in our cohort. Similarly, the associations with the molecular profiles of the *KRAS*, *NRAS*, and *BRAF* genes in this study also showed no statistically significant differences. Based on the data obtained from our study population of patients with advanced CRC, it was not possible to associate the presence of TILs with tumour molecular characteristics.

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Author contributions

(I) Conception and design: G Remonatto, LM Kliemann (II) Administrative support: E Ferreira Salles Pilar, G Remonatto; (III) Provision of study materials or patients: G Remonatto, F Paris; (IV) Collection and assembly of data: G Remonatto; (V) Data analysis and interpretation: G Remonatto, PG Schaefer (VI) Manuscript writing: All authors; (VII) Final approval of manuscript: All authors.

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Data availability

The authors chose not to share the data of the patients included in the study because of the General Data Protection Law (Law No. 13.709/2018) in force in Brazil. The patients consented to the use of their biological samples and associated data only for the purposes of this research.

Declarations

Ethical approval

The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was approved by the Research Ethics Committee of the Hospital de Clínicas de Porto Alegre, under CAAE (Certificate of Presentation for Ethical Consideration) number 56230122200005327. Patients included in the study consented to the use of their samples.

Conflict of interest
The authors have no conflicts of interest to declare.

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