The Influence of the Genetic and Immunologic Context in the Development of Colorectal Adenoma: A Case Series Report

A Influência do Contexto Genético e Imunológico no Desenvolvimento do Adenoma Colorrectal: Uma Série de Casos

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ABSTRACT

Introduction: Overcoming immunosurveillance is a major step in the progression of many types of tumors. Several immune escape strategies have been identified, including immunoediting and the establishment of an immune suppressive microenvironment. The aim of the present study was to determine whether the hereditary or sporadic context has any influence in the relationship between immune surveillance and tumor development, using sporadic and familial adenomatous polyposis related colorectal adenomas as a model.

Material and Methods: The immune tumor-infiltrating cells of a total of 58 low-grade and 18 high-grade colorectal adenomas were examined and compared, using immunostaining for CD3, CD4, CD8, CD57, CD68 and FoxP3.

Results: FoxP3 and CD68 counts were significantly higher in sporadic low-grade dysplasia (p = 0.0003 and p = 0.0103, respectively), and FoxP3 and CD4 counts were found to be significantly higher in high-grade sporadic dysplasia (p = 0.0008 and p = 0.0018, respectively) when compared with corresponding lesions in patients with familial adenomatous polyposis.

Discussion: This study suggests that the immune microenvironment of sporadic and hereditary lesions is different. Sporadic lesions contain a higher number of immune suppressive Treg cells, which suggests a stronger immune selective pressure. In contrast, hereditary lesions seem to benefit from a more tolerant immune microenvironment, allowing for the development of lesions with lower immune cell infiltration.

Conclusion: This study shows that sporadic lesions harbor higher tumor-infiltrating immune cell counts, which might reflect a higher immune tolerance towards hereditary lesions.

Keywords: Adenoma/diagnosis; Adenoma/genetics; Colorectal Neoplasms/diagnosis; Colorectal Neoplasms/genetics; Immunohistochemistry

RESUMO

Introdução: A capacidade de contornar a imunovigilância é fundamental na progressão de muitos tumores. Já foram identificadas várias estratégias de escape imunológico, incluindo *immunoediting* e o estabelecimento de um microambiente imunológico supressivo. O objetivo do presente estudo passa por determinar se o contexto hereditário ou esporádico influencia a relação entre a imunovigilância e o desenvolvimento do tumor, usando adenomas coloretais esporádicos e hereditários, no contexto de polipose adenomatosa familiar, como modelos.

Material e Métodos: Os infiltrados imunológicos tumorais de um total de 58 adenomas coloretais de baixo grau e de 18 de alto grau foram avaliados e comparados, usando imunohistoquímica com marcação para CD3, CD4, CD8, CD57, CD68 e FoxP3.

Resultados: As contagens celulares com imunorreatividade para FoxP3 e CD68 foram significativamente mais elevadas na displasia esporádica de baixo grau (p = 0,0003 e p = 0,0103, respetivamente), enquanto que as contagens para FoxP3 e CD4 foram significativamente mais elevadas na displasia esporádica de alto grau (p = 0,0008 e p = 0,0018, respetivamente) quando comparadas com lesões correspondentes em doentes com polipose adenomatosa familiar.

Discussão: O presente estudo sugere que o microambiente imunológico de lesões esporádicas e hereditárias é diferente. As lesões esporádicas contam com um número superior de células T reguladoras, supressoras da função imunológica, sugerindo-se uma pressão imune seletiva mais forte. Por seu turno, as lesões hereditárias parecem beneficiar de um microambiente imunológico mais tolerante, permitindo o desenvolvimento de lesões com menor infiltrado celular imune.

Conclusão: Este estudo demonstra que as lesões esporádicas contam com contagens de infiltrados imunológicos tumorais superiores, o que poderá refletir uma maior tolerância imunológica face a lesões hereditárias.

Palavras-chave: Adenoma/diagnóstico; Adenoma/genética; Inmunohistoquímica; Neoplasias Colorretais/diagnóstico; Neoplasias Colorretais/genética

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INTRODUCTION

Colorectal cancer (CRC) is the third most common cancer in men and the second in women.[1] Despite recent improvements, which include the introduction of new chemotherapeutic agents, the outcome for most patients remains relatively poor.[2,3] Therefore, additional treatment options beyond standard therapies (including surgery, chemotherapy and radiotherapy) are needed. There is renewed interest in immunotherapeutic options as there is a growing body of evidence supporting the existence of cancer immunosurveillance.[4]

Immunosurveillance exerts an important influence in tumor progression and in the efficacy of anti-cancer therapies.[5] Its success depends on the ability to detect neoantigens, frequently generated through mutations. Whether tumor-infiltrating lymphocytes represent the result of an inflammatory response that facilitates tumor progression, or a protective host response remains controversial.[4]

The presence of a pronounced lymphocytic infiltration within the tumor is associated with improved survival[6-14] and several studies have indicated a relationship between the number of infiltrating tumor CD8+ T lymphocytes and an improved prognosis in CRC.[15-18] Furthermore, the presence of high levels of tumor infiltrating memory T cells within CRC tissue has been associated with the absence of early metastatic spread and an improved disease-free and overall survival.[19] Moreover, the amount of activated intraepithelial CD8+ lymphocytes infiltration in CRC is related to stage, which might indicate that immune reaction is more prominent in early stages of disease and might have a stage specific influence on survival.[20,21] It is also currently known that immune dysregulation is already present in the early stages of the adenoma-CRC sequence.[22]

There are multiple pathways in colorectal cancer pathogenesis, which include the chromosomal instability (CIN), microsatellite instability (MSI) and CpG island methylator phenotype (CIMP) pathways.[23] CIN pathway is particularly suited for this study, since it includes, in most cases, a systematic progression through precursor lesions, which allows the study of the immune system activity in different stages of normal, precancerous and cancerous lesions of the colon. A key early mutation in the chromosomal instability (CIN) pathway is the mutation of adenomatous polyposis coli (APC) tumor suppressor gene. Familial adenomatous polyposis (FAP) is an autosomal dominantly inherited syndrome caused by germline mutations in the APC gene that accounts for approximately 1% of all CRCs.[24] Affected individuals develop hundreds to thousands of adenomas in the colon and rectum at unusually young ages. If left untreated, one or more adenomas progress invariably to CRC.

Inherited genetic variants have been demonstrated to affect both baseline and induced host immune responses. In fact, anti-tumor immune responses are intrinsic characteristics, controlled, in part by the host's genome.[25-27] However, such characteristics have yet to be systematically studied in hereditary models of cancer. A similar line of thought can be followed for somatic genetic variants. Since sporadic lesions typically originate much later (10 - 30 years) than hereditary lesions, one would predict the baseline mutation burden to be higher in sporadic lesions. This in turn, should translate into higher immunogenicity in sporadic lesions when compared with hereditary lesions.

The aim of the present study, using sporadic and FAP-related colorectal adenomas as a model, was to determine whether the hereditary or sporadic context has any influence in the relationship between immune microenvironment and tumor development.

MATERIAL AND METHODS

Case selection

A total of 58 patients were selected from Centro Hospitalar Universitário São João, Porto, Portugal who had undergone either resection or biopsy (one single case). Twenty-seven FAP cases (14 male and 13 female), diagnosed between 1999 and 2015, and 31 sporadic cases (16 male and 15 female), diagnosed between 2016 and 2018 were used. Normal mucosa, low-grade dysplasia and high-grade dysplasia were analyzed. All lesions were located in the colorectum, with the exception of one lesion located

Table 1 – Immunohistochemistry (IHC) protocol and staining patterns

Antibody	Type/Clone of primary Antibody	CC1 solution's exposure time (minutes)*	Option solution's exposure time (minutes)**	Antibody's exposure time (minutes)	Staining pattern
Anti-CD3	Anti-CD3 (2GV6) Rabbit monoclonal primary antibody	40 (95 °C)	Not used	20 (0.4 µg/ml)***	Cytoplasmic and membranous
Anti-CD4	Anti-CD4 (SP35) Rabbit monoclonal primary antibody	40 (95° C)	4	32 (2.5 µg/ml)***	Membranous
Anti-CD8	Anti-CD8 (SP57) Rabbit monoclonal primary antibody	24 (100° C)	4	0 (0.35 µg/ml)***	Membranous
Anti-CD57	Abcam monoclonal mouse AB 187274	40	8	28 (1:300 dilution)	Cytoplasmic and membranous
Anti-CD68	Anti-CD68 (KP-1) Primary Antibody	16 (100° C)	8	16 (0.4 µg/ml)***	Cytoplasmic and membranous
Anti-FoxP3	Polyclonal anti-FoxP3 rabbit NB 600-245	40	4	40 (1:300 dilution)	Nuclear

*: CC1: Cell conditioning 1 is a prediluted solution used as a pretreatment step in IHC tissue processing reactions carried out in Ventana™ BenchMark; **: Prediluted solution ready-to-use in order to diminish background staining; ***: Prediluted antibody solution.

in the duodenum. Lesions examined in PAF cases were mostly of tubular architecture (14/27), followed by tubulovillous (4/27), villous (2/27) and tubulopapillary (1/27), whilst sporadic lesions were predominantly of tubulovillous architecture (22/31), followed by tubular (7/31) and villous (1/31) varieties.

Patients were matched for gender, but not for age, since CRC pathogenesis occurs at younger ages in FAP patients.

In accordance with Article 19 of Portuguese Law No. 12/2005 of January 26, no ethical consent was necessary for this study because it was based on the use of retrospective human samples.

Immunohistochemical staining

Tissue sections were obtained from selected paraffin-embedded blocks, which were serially cut with a microtome (microm HM325) at 2 µm thickness and mounted on positively charged microscopic slides (Superfrost Plus™ from Thermo Scientific™). Amygdala was used as a positive control on each slide. Deparaffinization was accomplished with Ezprep at 72°C over 20 minutes. Immunohistochemistry (IHC) staining was performed with the Ventana™ BenchMark ULTRA system. An optimization protocol, approved by a senior pathologist and a lab technician, was performed to achieve appropriate staining conditions. The protocol used is shown in Table 1.

Primary antibodies against CD3, CD4, CD8 and CD68 were added automatically by the Ventana™ machine with the aforementioned protocols, whilst FoxP3 and CD57 respective primary antibodies were added manually. OptiView™ Universal DAB detection kit was used.

Haematoxylin was used as a contrast agent (1 x 8 minutes) followed by Bluing reagent, an after-contrast agent (1 x 4 minutes).

After IHC, slide washing was performed with Ezprep (1 x 10 minutes), detergent and running water (1 x 10 minutes until clean), followed by dehydration with ethanol [70% (1 x 10 minutes), 95% (1 x 10 minutes) and 100% (1 x 10 minutes)] and xylene (2 x 10 minutes). Finally, the slides were mounted with Entellan and a protective slide.

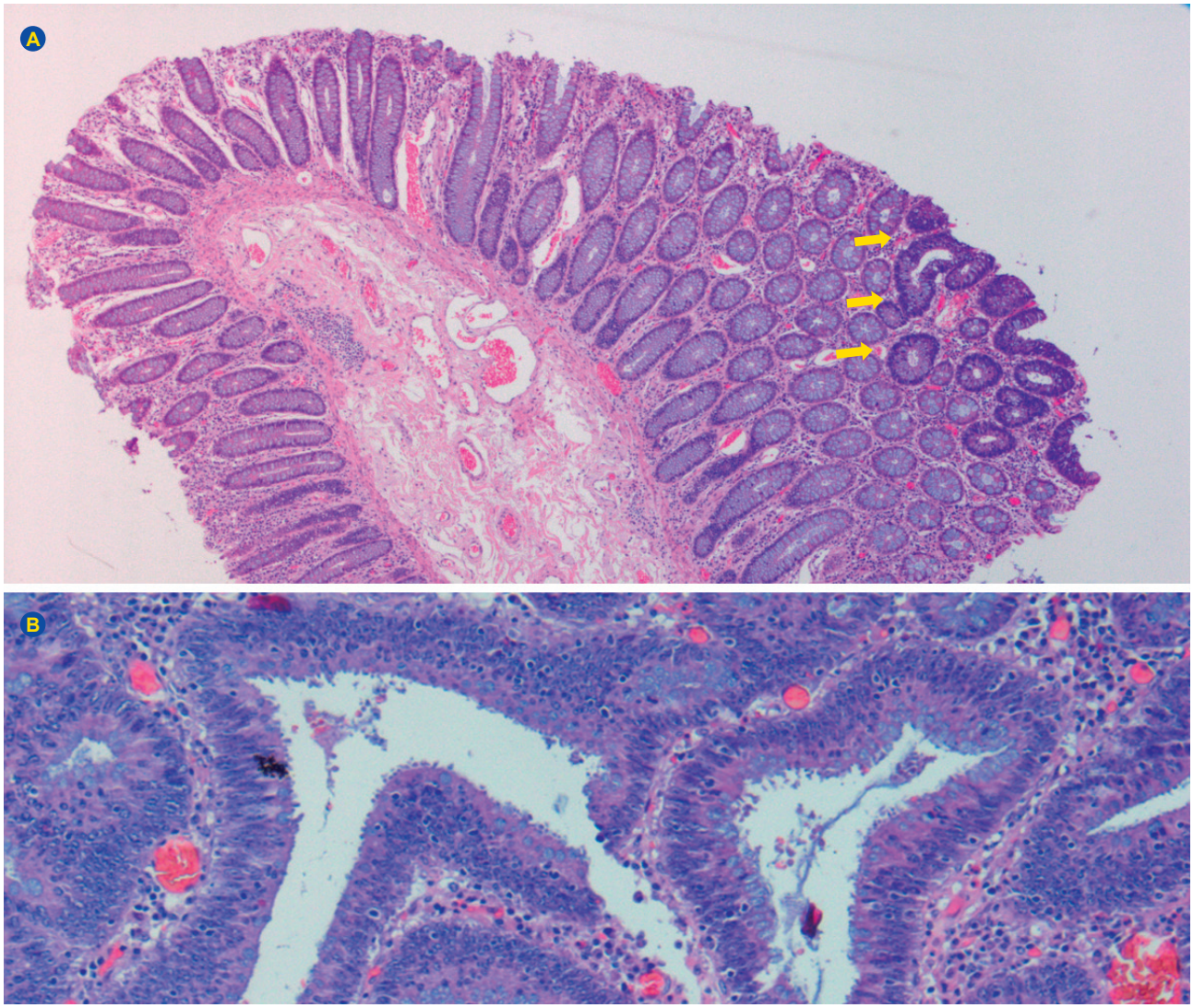

Figure 1 – (A) Normal mucosa (x 4) (left side) and low-grade dysplasia in a familial adenomatous polyposis patient (x 4) (arrows); (B) High-grade dysplasia in a familial adenomatous polyposis patient (x 10).

Slide analysis

Haematoxylin and eosin (H&E) histological slides of each case were reviewed together with a senior pathologist to identify normal mucosa and adenomatous lesions. For each H&E case, a total of six IHC stained slides were obtained (one slide for each different primary antibody). For each of the IHC slides a total of eight high quality digital color images of the areas were obtained at 40x magnification using an Olympus BX 43 microscope (camera Olympus DP73) (four images of normal mucosa and four images of the dysplastic lesion) for a total of 48 images per case. An example of normal mucosa can be observed in Fig. 1A, of low-grade dysplasia in Fig. 1A and of high-grade dysplasia in Fig. 1B.

Each image representing a High-power field (HPF), corresponding to 0.06 mm^2 was used to count the stained cells. Individual cells were manually counted by two observers in a double-blind setting, using ImageJ software (National Institutes of Health) that counts the clicks on each cell (cell counter plugin). The entire image was used for counting.

Inflammatory cells within areas of hemorrhage, necrosis, blood vessels, nerves or large lymphoid aggregates were not quantified.

Cells were counted on each of the four images of both normal and adenomatous tissue, corresponding to a total area of 0.24 mm^2, respectively.

Statistical methods

For low-grade dysplasia, the differences in the number of stained cells/mm^2 between normal tissue and lesions were evaluated using unpaired Student's *t*-tests; the difference between the number of stained cells/mm^2 between hereditary and sporadic lesions was tested using unpaired Student's *t*-test.

Regarding high-grade dysplasia, due to the small sample size (n = 9) and the presence of several outliers, non-parametric tests were used to infer the difference in the number of stained cells/mm^2 between normal tissue and lesions (Wilcoxon matched-pairs signed rank test); the difference between the number of stained cells/mm^2 between hereditary and sporadic lesions was tested using a Mann Whitney test.

RESULTS

Tumor-infiltrating immune cells

The tumor-infiltrating cells of a total of 58 cases of low-grade dysplasia (Fig. 2A) and 18 cases of high-grade dysplasia were examined and compared (Fig. 2B). Immunoreactivity to CD3, CD57 and CD68, exhibiting a cytoplasmic and membranous pattern, to CD4 and CD8, exhibiting a membranous staining pattern and to FoxP3, exhibiting a nuclear staining pattern were analyzed. Immune cell counts were found to be generally higher in the margin than in the tumor in both high-grade and low-grade dysplasia and in both hereditary and sporadic contexts.

Admitting a significance level of 5%, FoxP3 and CD68-positive cell counts were significantly higher in low-grade dysplasia in patients with sporadic CRC (p = 0.0003 and p = 0.0103, respectively) when compared with corresponding lesions in FAP patients, whilst CD3, CD4, CD8 and CD57 counts were similar. In high-grade dysplasia lesions, CD4 and FoxP3 counts were significantly higher in patients with sporadic CRC (p = 0.0008 and p = 0.0018, respectively) when compared with corresponding lesions in FAP patients, whilst CD3, CD8, CD57 and CD68 where similar. Examples of low-grade adenomas from both sporadic and FAP patients can be observed in Figs. 3A to 3D, whilst examples of high-grade adenomas can be observed in Figs. 4A to 4D. The comparative analysis of cell counts in familial adenomatous polyposis (FAP) and sporadic (SPO) adenomas is shown in Table 2.

DISCUSSION

Accumulating evidence supports the importance of the host's immune system on the development of cancer,[7,8,28-32] which may offer powerful prognostic tools and guide the need for systemic therapy. Immunosurveillance can not only eliminate tumors but also select variant tumor cells that can

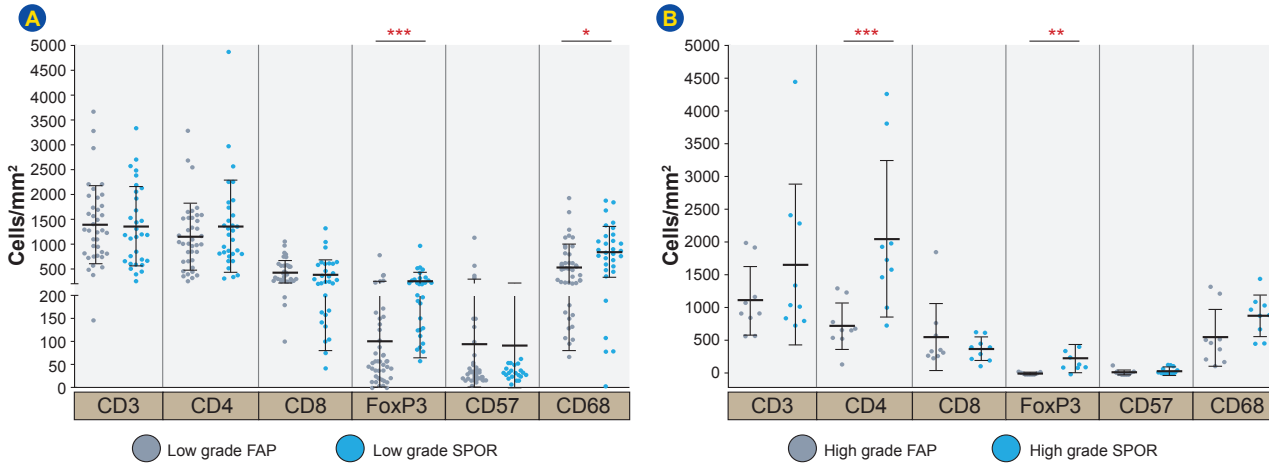

Figure 2 – (A) Low grade dysplasia tumor-infiltrating cells; (B) High-grade dysplasia tumor-infiltrating cells

*$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$; ****$p \leq 0.0001$; ns $p > 0.05$

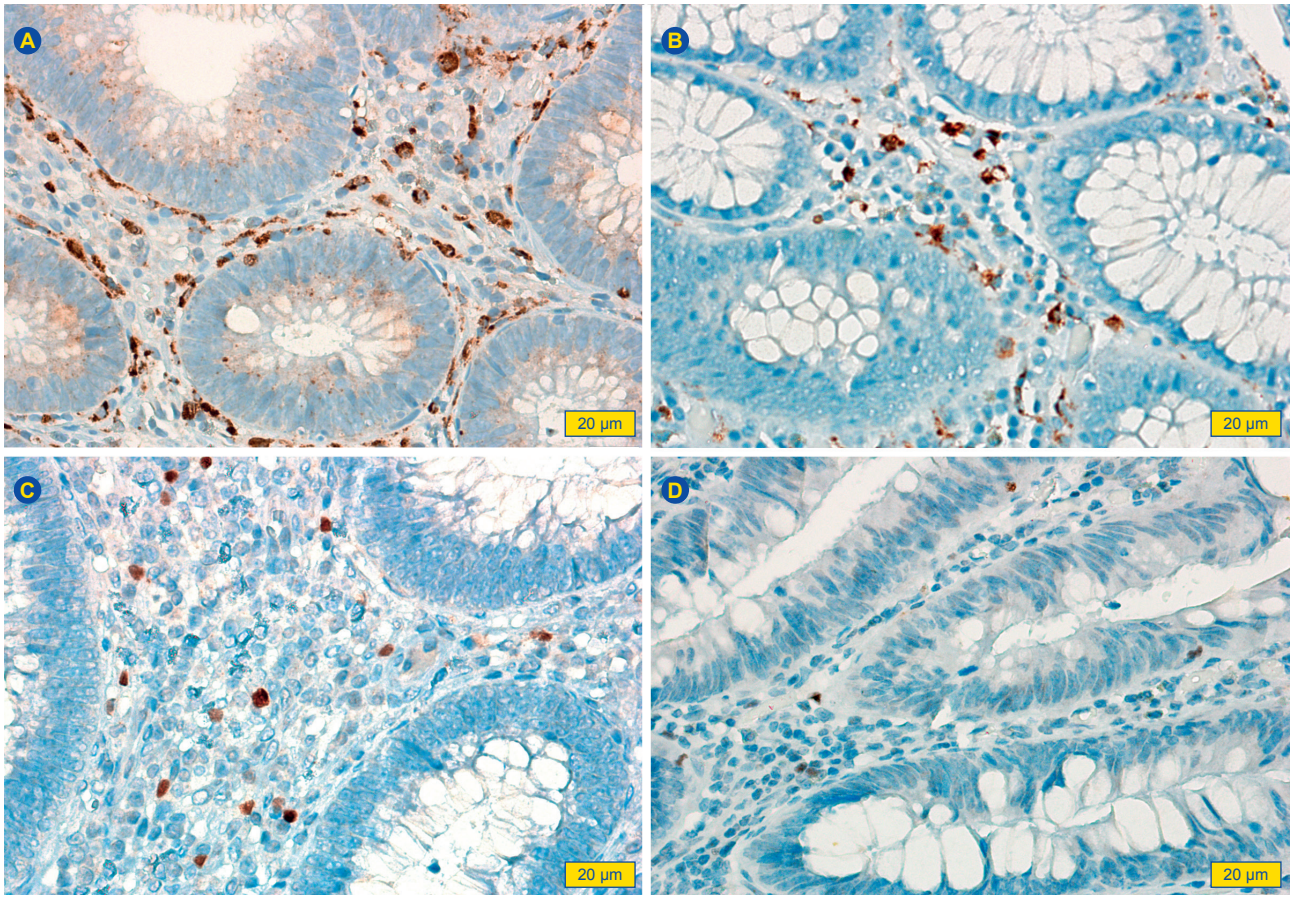

Figure 3 – Low-grade dysplasia (x 40): CD68 stained cells in sporadic (A) and familial adenomatous polyposis (B) adenoma; FoxP3 stained cells in sporadic (C) and familial adenomatous polyposis adenoma (D).

resist it, a process called 'immunoediting'.[33,34] Higher densities of tumor-infiltrating lymphocytes are generally associated with improved clinical outcome.[35] In particular, higher densities of CD3+ T cells and CD8+ T cells have been associated with longer disease-free survival and/or improved overall survival. Therefore, the role of the adaptive immune response is becoming increasingly appreciated. On the other hand, there is experimental evidence supporting the idea that the innate immune system can promote tumor development through inflammation-dependent mechanisms.[36,37] Therefore, the precise analysis of the tumor microenvironment by pathologists may be essential for future clinical implementation and better patient management.

Previous studies have examined the independent prognostic effect of tumor-infiltrating immune cells in CRC models, but as far as we know, this is the first study that examines the implication of the hereditary context of FAP in the tumor-infiltrating cells of pre-cancerous lesions.

The current study demonstrates that the hereditary context of FAP results in significantly different phenotypes of tumor-infiltrating immune cells. In sporadic low-grade dysplasia, a significantly higher number of FoxP3 and CD68 stained cells was observed, whilst CD4 and FoxP3 cell counts were significantly higher in sporadic high-grade dysplasia lesions.

Regulatory T cells (Treg) are a subpopulation of CD4+ T cells that express the transcription factor forkhead box protein P3 (FoxP3) and hold a key role in maintaining self-tolerance by suppressing the activation and function of self-reactive lymphocytes.[38] In cancer infiltrates, however, Treg cells suppress the induction and proliferation of effector cells to impair antitumor immunity, contributing to a poor clinical outcome in cancers with abundant Treg infiltration. However, its role in CRC is controversial, having a potentially positive impact by controlling cancer-driving inflammation, whilst, also, promoting tumor progression by impeding specific immune responses.[39] In fact, higher densities of Treg cells have been associated with better survival in CRC,[40,41] which might explain the higher counts of FoxP3 stained cells in sporadic lesions, observed in both low grade and high grade dysplasia in the current study.[42]

Tumor-associated macrophages (TAMs), stained with CD68, have been associated with a pro-tumor phenotype in both primary tumors and metastasis, by promoting increased growth, angiogenesis, metastases, immunosuppression and poorer differentiation.[43,44] Its role in CRC, however, is not as straightforward, and, in contrast to most other malignancies, in CRC, increased macrophage infiltration is associated with better patient prognosis.[12,45-50] It is likely that infiltrating monocytes are primed in the tumor micro-environment to exert either anti or pro tumorigenic characteristics, depending on environmental cues that they receive, with most studies on colon cancer supporting a tumor-inhibiting role of macrophages. Therefore, higher

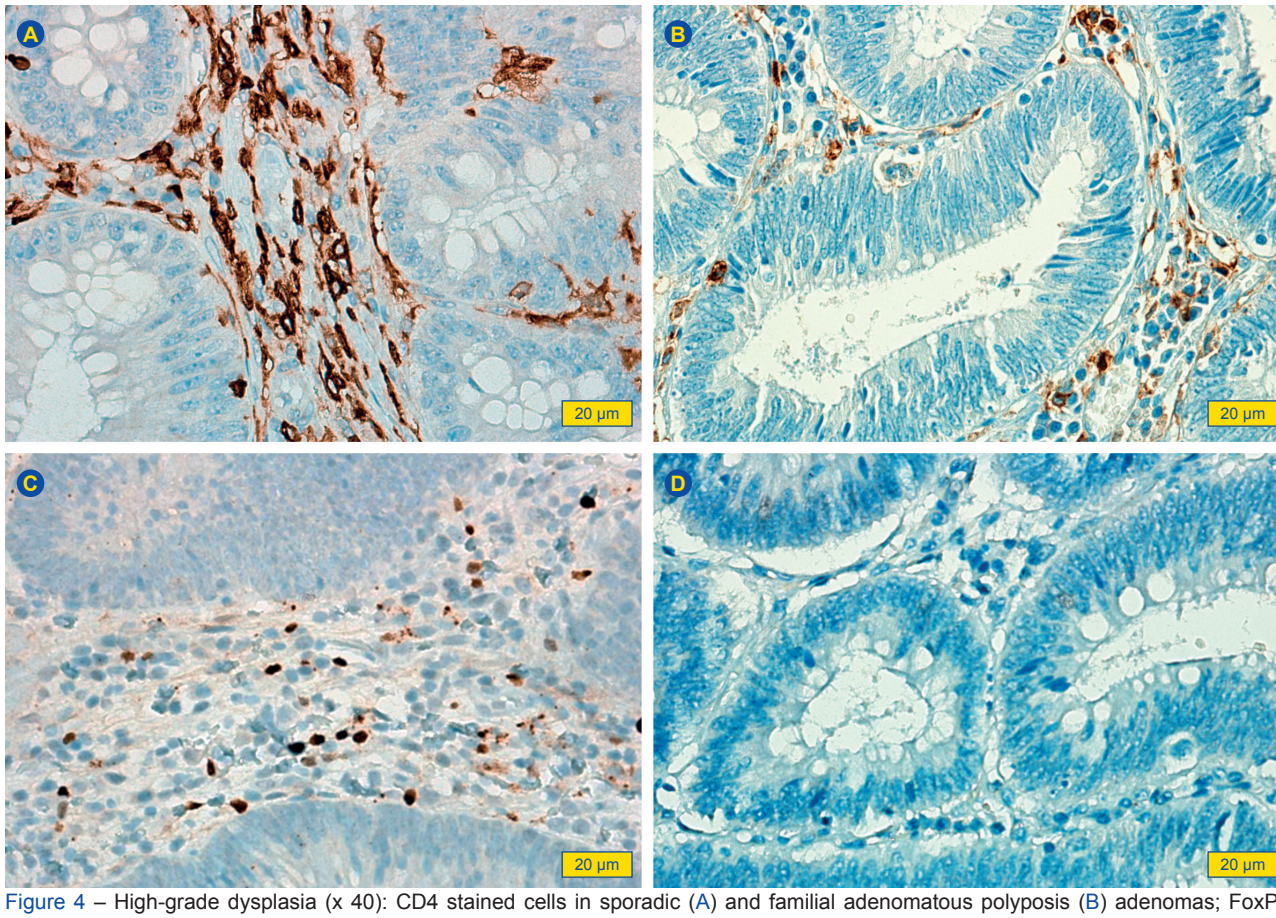

Figure 4 – High-grade dysplasia (x 40): CD4 stained cells in sporadic (A) and familial adenomatous polyposis (B) adenomas; FoxP3 stained cells in sporadic (C) and familial adenomatous polyposis adenomas (D).

Table 2 – Comparative analysis of cell counts in familial adenomatous polyposis (FAP) and sporadic (SPO) adenomas

Low grade dysplasia *(FAP versus SPO)*	**FAP cell counts** (mean)	**Sporadic cell counts** (mean)	***p*-value**	**Statistical significance**
CD3	1397	1353	0.8178	ns
CD4	1152	1351	0.3042	ns
CD8	438	384.3	0.4033	ns
FoxP3	100.3	246.8	0.0003	***
CD57	94.92	91.13	0.9298	ns
CD68	538	842.9	0.0103	*
High grade dysplasia *(FAP versus SPO)*	**FAP cell counts** (median)	**Sporadic cell counts** (median)	***p*-value**	**Statistical significance**
CD3	925	1050	0.4363	ns
CD4	675	742	0.0008	***
CD8	354.2	412.5	0.6665	ns
FoxP3	4.167	137.5	0.0018	**
CD57	20.83	25	0.5560	ns
CD68	475	900	0.1135	ns

*: $p \leq 0.05$; **: $p \leq 0.01$; ***: $p \leq 0.001$; ****: $p \leq 0.0001$; ns: $p > 0$.

infiltrating densities are generally associated with decreased recurrence, diminished metastases and increased patient survival,[45,47-49,51,52] which might help to explain why significantly higher counts were identified in the current study in sporadic lesions, specifically in low grade lesions, implying that its anti-tumor effect might be lost during the progression from low grade to high grade lesions.

Tumor-selective infiltration of CD4+ T cells might be explained by the role played by CD4+ cells during the primary antigen-specific response in imprinting CD8+ T cells with the ability to develop into long-living memory cells,[53] which might explain the higher counts observed in this study in high grade dysplasia lesions of sporadic patients.

CONCLUSION

This study shows that sporadic lesions harbor higher tumor-infiltrating immune cell counts, which might reflect a higher immune tolerance towards hereditary lesions. Although these results need to be independently confirmed, it is tempting to speculate that immune tolerance may also help explain the earlier onset and multifocal pattern typically observed in hereditary lesions.

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PROTECTION OF HUMANS AND ANIMALS

The authors declare that the procedures were followed according to the regulations established by the Clinical Research and Ethics Committee and to the Helsinki Declaration of the World Medical Association.

DATA CONFIDENTIALITY

The authors declare having followed the protocols in use at their working center regarding patients' data publication.

CONFLICTS OF INTEREST

The authors declare that they have no conflict of interest with regard to this article.

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