

G-protein coupled short-chain fatty acid receptor GPR 43 fluorescence immunohistochemistry staining protocol for confocal microscopy in human colon cancer

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ABSTRACT

Background: G-protein coupled receptor 43 (GPR43) mediates the biological effects of short-chain fatty acids (SCFAs) in the colon, but its role in colon cancer (CC) remains insufficiently characterized. This pilot study presents in detail a cost-effective and easily reproducible fluorescence immunohistochemistry protocol for staining GPR43 in formaldehyde-fixed, paraffin-embedded human colon tissue.

Methods: Using samples from patients with colon adenocarcinoma (ACC) and signet ring cell carcinoma (SRCC), we optimized staining procedures with generic reagents and a custom 3D-printed slide tray, facilitating minimal reagent usage and consistent sample coverage.

Results: Confocal microscopy revealed distinct GPR43 localization on the apical and basal membranes of colonocytes and goblet cells in healthy tissue. In contrast, ACC tissue exhibited diffuse, disorganized staining, while SRCC samples showed minimal receptor expression. These findings suggest a link between GPR43 expression patterns and tissue differentiation status. Additionally, we observed autofluorescence at 488 nm of eosinophils without staining, as previously reported in literature.

Conclusions: These findings may support prognostic assessments based on intratumoral or peritumoral eosinophil counts. The present study shows in detail an easily reproducible IHC staining method for GPR 43 in paraffin embedded tissue samples, using only generic materials and highlights its potential role as a biomarker or therapeutic target in CC.

Keywords: colon cancer, confocal microscopy, GPR43, immunohistochemistry, short-chain fatty acids

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INTRODUCTION

Colon cancer (CC) is a major public health issue and one of the leading causes of cancer-related hospitalization and death globally [1]. Histologically, over 90% of CC is adenocarcinoma (ACC), with its origin in the glandular epithelium, which can be further categorized into well-differentiated, moderately differentiated, or poorly differentiated ACC depending on the proportion of glandular tissue within the tumor [2].

Other types of CC include, but are not limited to, signet ring cell, mucinous, medullary, micropapillary, serrated, cribriform, adenosquamous, spindle cell, and completely undifferentiated CC. The incidence of these types of CC is relatively rare compared to ACC [2,3]. Signet ring cell carcinoma

(SRCC) represents <1% of all CCs, being more characteristic of stomach cancers. The tumor volume is comprised of >50% of characteristic signet ring cells, with a large mucin-containing vacuole that displaces the nucleus of the cell to the periphery [4]. Due to its poor differentiation, it usually presents a worse outcome compared to other types of ACC [2,3].

Short-chain fatty acids (SCFAs) are carboxylic acids that contain up to five carbon atoms, the three main SCFAs being acetate (Ac), propionate (Prop), and butyrate (Bu). Among these, Ac and Prop are absorbed from the colon almost completely and are found in significant quantities in portal blood as well as various tissues throughout the body, while Bu remains primarily in the lumen of the colon [5]. Microbial fermentation of indigestible food fibers in the gut is the primary source of SCFAs [5,6].

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Several effects have been attributed to SCFAs, the essential being increased leptin production [7]. The majority of these effects are accomplished through two G-protein coupled receptors, GPR 41 also known as free fatty acid receptor 3 (FFAR 3), and GPR 43, also known as free fatty acid receptor 2 (FFAR 2), which are primarily found in adipose tissue and colon [7,8].

Among all the SCFAs, Bu is absorbed the least from the colon and is found in the highest concentration in the lumen. It represents one of the main energy substrates of colon epithelial cells [9,10]. Butyrate, together with Prop, has also been shown to have the potential to prevent CC by regulating the division and proliferation of the colon epithelium [10,11].

While there are studies that have shown the expression of SCFA receptors in human colonic tissue [8,12], there is sparse evidence for their expression in CC [13]. Butyrate and Prop have antitumoral effects on the colon epithelium, and the normal colon epithelium expresses SCFAs receptors; however, there is slight evidence regarding the presence and localization of SCFAs receptors in neoplastic colon tissue [10,11].

As a validated technique for highlighting the presence of receptors produced by tumor cells, fluorescence immunohistochemistry (IHC) is classically carried out on frozen tissues sliced with a cryotome, avoiding the use of formaldehyde as a fixator because it modifies the antibody epitopes in tissues, preventing the binding of primary antibodies. Formaldehyde-fixed and paraffin-embedded tissue blocks, commonly used in the clinical pathology lab, are widely available, easily processed, and present long-term stability; however, they require special processing for successful staining.

The present pilot study aims to establish a simple, easy-to-follow, cost- and time-effective sample processing and fluorescence-based IHC staining protocol for GPR 43 used for examination at the confocal microscope using standard formaldehyde-fixed and paraffin-embedded human colon tissue, as well as to analyze the presence and localization of GPR 43 receptor in neoplastic versus normal colon epithelium.

METHODS

Study design

Formaldehyde-fixed and paraffin-embedded tissue blocks from three patients histopathologically diagnosed with CC and admitted for colectomy were selected from the pathology archive of “Prof. Dr. Octavian Fodor” Regional Institute for Gastroenterology and Hepatology Cluj-Napoca. The tissue blocks were obtained from the surgical specimens and selected to include both healthy and

malignant tissue on the same block. All blocks were collected from patients admitted in October 2023.

This study was performed in accordance with the Declaration of Helsinki and approved by the Medical Ethics Committee of the “Iuliu Hațieganu” University of Medicine and Pharmacy, Cluj-Napoca, with the approval code AVZ 7/30.01.2024.

Sample collection and preliminary processing

The selected tissue blocks were sliced into 3 µm thick consecutive slices and deposited on Superfrost™ Plus adhesion microscope slides (Epredia, Netherlands). A total of three slides were obtained from each tissue block. After drying, the slides were deparaffinized using standard procedures. In summary, the slides were submerged successively for 3 minutes each in the following solutions in this order: 100% xylene, 1:1 xylene-ethanol mixture, 100% ethanol, 95% ethanol, 70% ethanol, 50% ethanol, and distilled water.

After deparaffinization, one slide per block was stained with hematoxylin and eosin (H&E) to facilitate visualization of structures, and two were reserved for immunohistochemical staining, stored short-term in ultrapure distilled water (DW). At no point after deparaffinization should the slides be left to dry, as this increases non-specific binding and/or background autofluorescence.

Immunohistochemical staining

During immunohistochemical slide processing, citrate antigen recovery buffer (CARB), tris-buffered saline (TBS), and triton x-100 wash solution (Tx) were extensively used from in-lab prepared stock solutions diluted as needed. The compositions of these stock solutions, the dilutions used, as well as the concentrations for the primary and secondary antibody (Abcam, Cambridge, United Kingdom) solutions are presented in Table 1. The fluorescent dye 4',6-diamidino-2-phenylindole (DAPI) (Sigma-Aldrich, Merck KGaA, Darmstadt, Germany) was also employed for the staining of cell nuclei. Normal donkey serum (Abcam, Cambridge, United Kingdom) was also employed for blocking of non-specific binding.

Antigen recovery was carried out using a CARB and a commercial vegetable steamer. A sufficiently large heat-resistant plastic container to completely cover all slides was inserted into the vegetable steamer, filled with CARB, and left to heat up to its working temperature of 100°C. After confirming the temperature of the CARB with a laboratory thermometer, the slides were loaded into a slide rack and inserted into the CARB.

After incubating for 20 minutes in the CARB, the slides were removed and immediately placed under cold, slowly running tap water for 10 minutes to cool. After cooling, the slides were rinsed with DW and placed in a clean slide staining vat filled with DW at room temperature.

After antigen recovery, the slide rack was removed from DW and placed in a container filled with Tx on a rocker shaker for five minutes. Afterwards, a fresh Tx solution was used to wash the slides for another five minutes.

The slides were removed from the Tx and transferred upside down to 3D-printed slide trays of our design, printed from generic polylactic acid filament for convenience and ease of printing (.3mf file for tray and corresponding lid available for download at: <https://www.printables.com/model/1367120-slide-staining-tray/related>). Each well was filled with 500 µl of diluted normal donkey serum, and the slides were incubated at 24°C for 1 hour, after which they were washed 2 times in Tx for

5 minutes each (Figure 1). Using a new tray, each well was filled with 500 µl of diluted primary antibody (Rabbit Anti-GPR 43) and the slides were placed on top. After covering the tray with the lid, it was placed in a dark container, onto wet tissue paper and kept overnight (for 14 hours) at 4°C. This slide tray and lid reduce the chance of the antibody solution drying out overnight and keep solution consumption to a minimum while maintaining the complete slide coverage necessary for samples with a large area.

The next morning, the slides were removed from the tray and washed two times in Tx as previously described, then transferred to a new tray containing the diluted secondary

Table 1. Preparation of stock and working solutions used during sample processing

Solution	Stock solution	Working solution
CARB	900 mL DW + 29.4 g (100 mmol) sodium citrate dihydrate + 5 mL Tween 20 Adjust to pH=6.0 using 1M NaOH Add DW to a final volume of 1000 mL	Add 100 mL of stock solution to 900 mL DW (10x dilution) for a working solution of 10 mM sodium citrate, 0.05 % Tween 20
TBS	800 mL DW + 88 g (1.5 mol) NaCl + 24.2 g (200 mmol) Tris Adjust to pH = 7.5-7.6 using 1M HCl Add DW to a final volume of 1000 mL	Add 100 mL of stock solution to 900 mL DW (10x dilution) for a working solution of 8.8% (150 mM) NaCl, 2.42% (20 mM) Tris
Tx	1000 mL TBS + 500 µL Triton X-100	Use as is
Donkey normal serum (ab7475)	100 mg/mL	Add 100 µL of serum to 900 µL TBS for a working concentration of 10%
Rabbit anti-GPR 43 (ab188909)	Concentration = 1 mg/mL	Add 5 µL of antibody solution to 2995 µL TBS for a working concentration of 2.5 µg/mL
Donkey anti-rabbit Alexa 488 (ab150065)	Concentration = 2 mg/mL	Add 3 µL of antibody solution to 2997 µL TBS for a working concentration of 2 µg/mL
DAPI (D9542)	Dissolve 1 mg/mL TBS	Add 10 µL stock solution to 990 µL TBS then from the resulting solution add 500 µL to 4500 µL TBS for a working concentration of 1 µg/mL

Abbreviations: CARB – citrate antigen recovery buffer, DW – distilled water, GPR – G-protein coupled receptor, TBS – Tris-buffered saline, Tx – Triton x-100 wash solution.

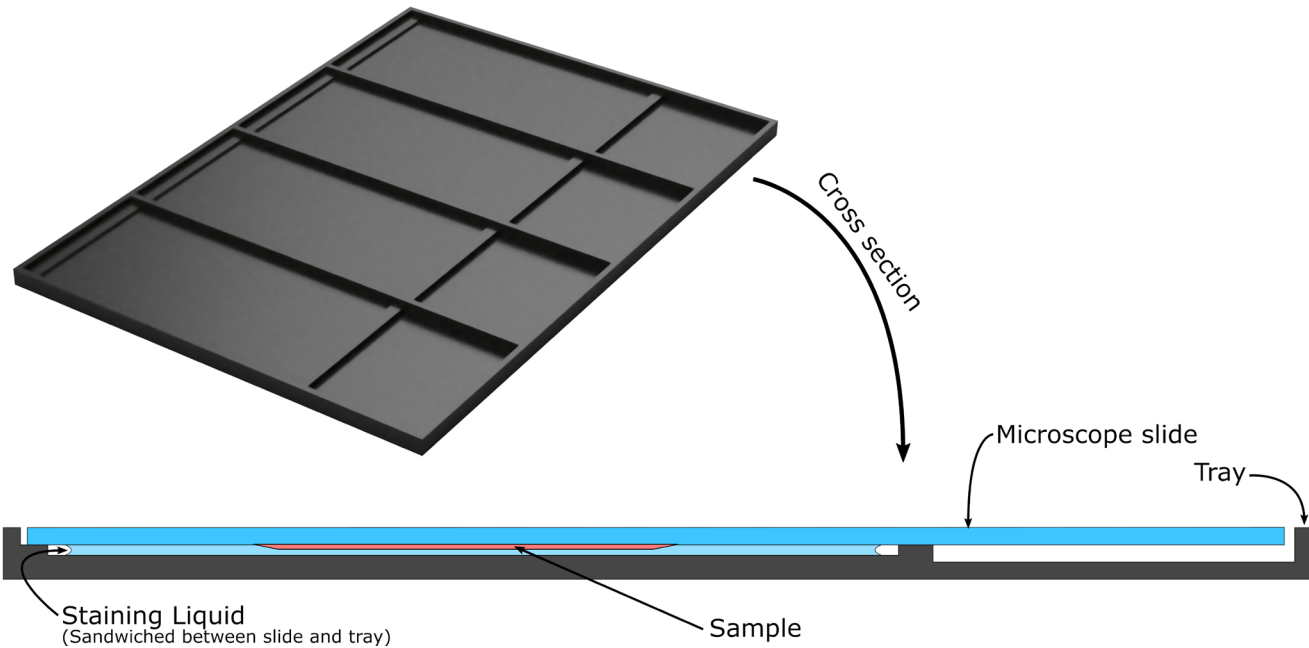


Figure 1. Design of 3D-printed slide staining tray with cross-section

The slide trays were 3D-printed from generic polylactic acid filament (.3mf file for tray and corresponding lid available for download at: <https://www.printables.com/model/1367120-slide-staining-tray/related>).

antibody (Donkey Anti-Rabbit Alexa 488) and placed in a dark container onto wet tissue paper for two hours at 24°C.

After secondary antibody incubation, the slides were removed from the tray and washed two times in TBS for five minutes, then transferred to a tray containing DAPI staining solution for 10 minutes.

After DAPI staining, slides were washed two times in Tx and then rinsed with DW. The entire staining process is summarized in Figure 2.

Sample analysis

After staining, the samples were analyzed unmounted, immediately, to avoid the slides drying out. If an immediate analysis is impossible, slides should be mounted using an aqueous fluorescence-compatible mounting medium and coverslips.

Confocal microscopy analysis was carried out using a Re-Scanning Confocal Microscope (RCM) (Confocal.nl, Amsterdam, The Netherlands) fitted to an Eclipse Ti2-E inverted microscope (Nikon, Amstelveen, The Netherlands). A 20x Plan Apochromat (N.A.=0.75) objective was employed, and the images were scanned using a 488 nm diode laser (for the Alexa 488 labeled antibodies for GPR43) and a 405 nm diode laser (for DAPI-stained nuclei). The images were captured using a CCD PCO EDGE 4.2 digital camera for the RCM-Vis module and were subsequently analyzed using the NIS Elements software (v5.30.04, Nikon). Image acquisition parameters were set and maintained across image acquisitions for stained and unstained specimens, as well as the negative control. Mean fluorescence intensity (MFI) was measured for whole images as well as sections of images, which are specified accordingly.

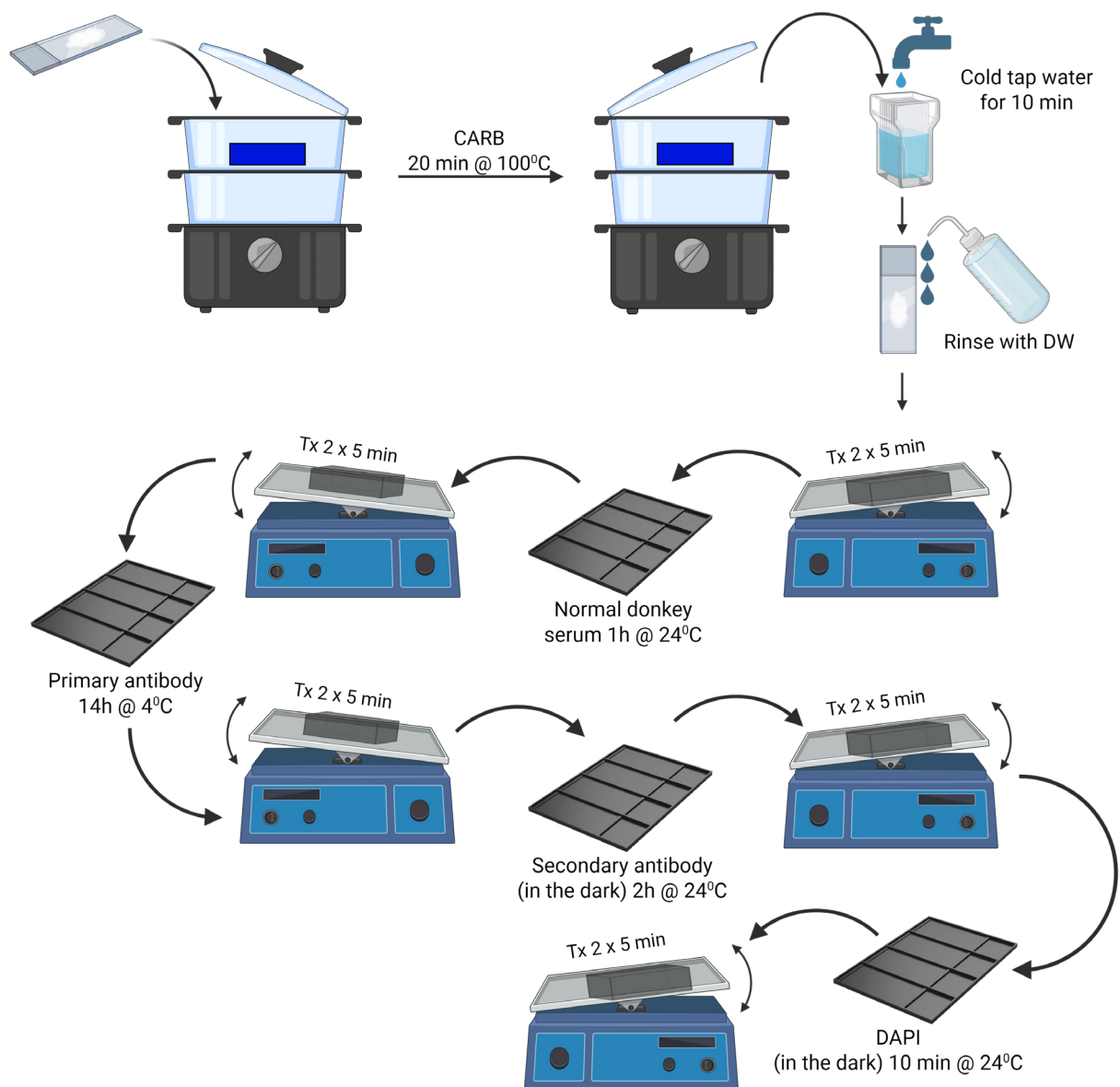


Figure 2. Summary of immunohistochemistry staining procedure

Abbreviations: CARB – citrate antigen recovery buffer, DW – ultrapure distilled water, Tx – Triton X-100 wash solution, DAPI – 4',6-diamidino-2-phenylindole.

RESULTS

Two patients had a positive histopathological diagnosis of well-differentiated ACC, and one had SRCC. These diagnoses were based on the routine histopathological examination and were retrieved from the lab reports for each patient.

GPR 43 staining was observed in almost all epithelial cells, as well as the lamina propria. This staining was not observed in the negative control, stained only with the secondary antibody (Figure 3).

Staining of the luminal side and basal side of normal colon epithelial cells appeared to be more intense compared to the lateral sides (Figure 4A). Meanwhile, goblet cells presented a distinct thin membrane-like appearance of the signal, with more intense staining towards the luminal side (Figure 4B).

The staining of epithelial cells was more intense compared to goblet cells and epithelial cells at the surface presented a more intense signal compared to epithelial cells from crypts.

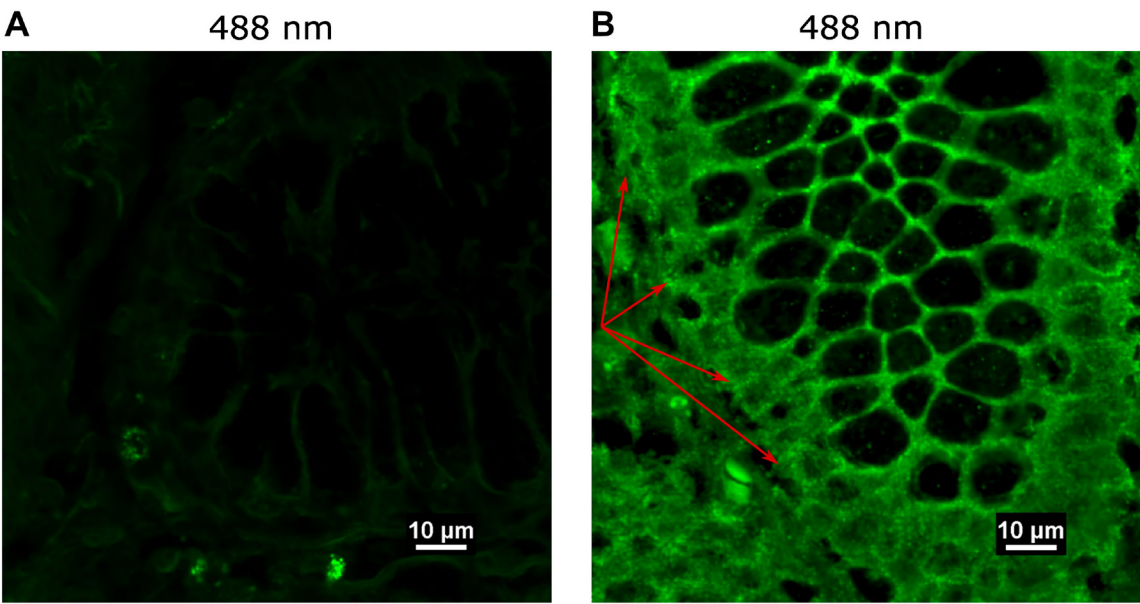


Figure 3. Immunofluorescence detection of GPR 43 in normal colon crypts
(A): Negative control (no primary antibody) Whole image MFI = 115.44. (B): Stained specimen (primary and secondary antibody) Whole image MFI = 175.59. Red arrows point to the lamina propria boundary.

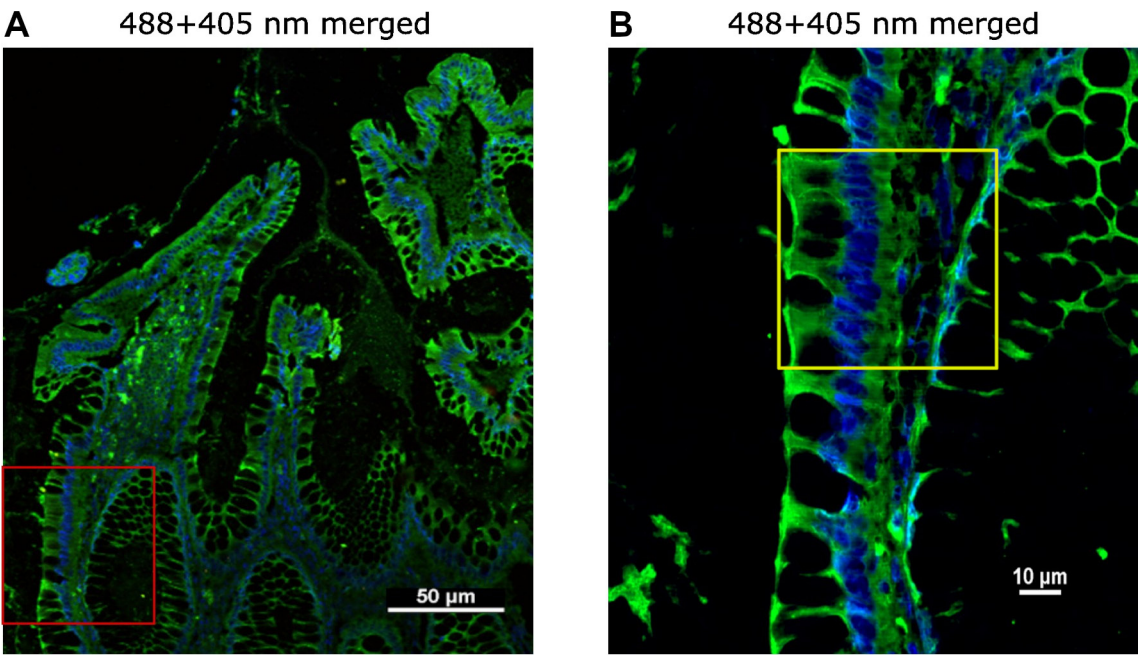


Figure 4. Immunofluorescence detection of GPR 43 in normal colon merged with DAPI stained nuclei
(A): Composite large image of normal colon tissue. (B): Detail of image from the left, of the area indicated by a red square. Yellow square MFI (488 nm channel only) = 567.46.

Adenocarcinoma tissue presented a more diffuse and punctate appearance compared to healthy tissue, with no specific cellular level localization of the signal (Figure 5). In the case of SRCC, staining was very weak, close to the negative control, and the localization was heterogeneous throughout the tissue (Figure 6).

Multiple analyzed areas contained large granular cells in the lamina propria and within invasive tumoral tissue that exhibited intense fluorescence upon stimulation at 488 nm. Similar signals were also observed in negative control slides incubated with secondary antibodies only,

suggesting that the fluorescence was not due to specific staining. The morphology of these cells was highly suggestive of eosinophils (Eos). To confirm this, an H&E-stained slide was examined in brightfield microscopy, allowing clear identification of eosinophils by their characteristic morphology. The microscope was then switched to confocal mode to analyze the same regions. The cells displayed strong fluorescence under 488 nm excitation, which was subsequently confirmed to represent autofluorescence of the cytoplasmic granules on a completely unstained specimen (Figure 7).

488+405 nm merged

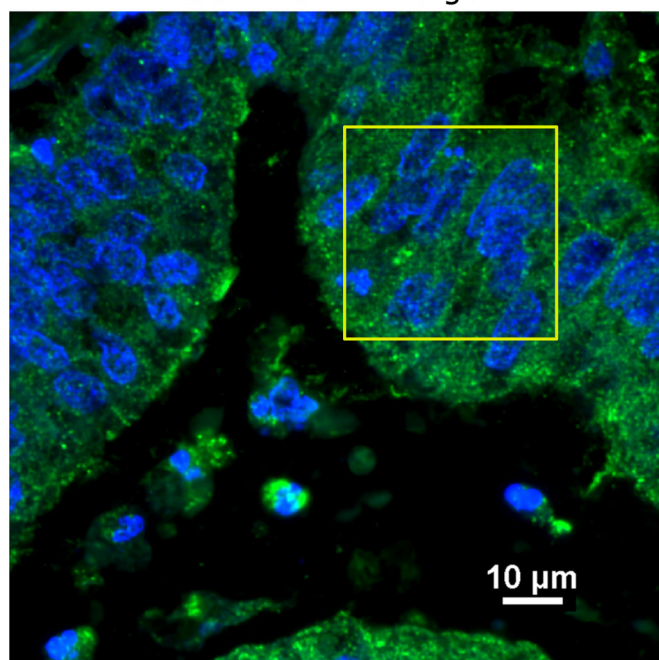


Figure 5. Colon adenocarcinoma (ACC) tissue

Alexa 488 labeled antibody stain for GPR 43 merged with DAPI-stained nuclei. Yellow square MFI (488 nm channel only) = 156.84.

488+405 nm merged

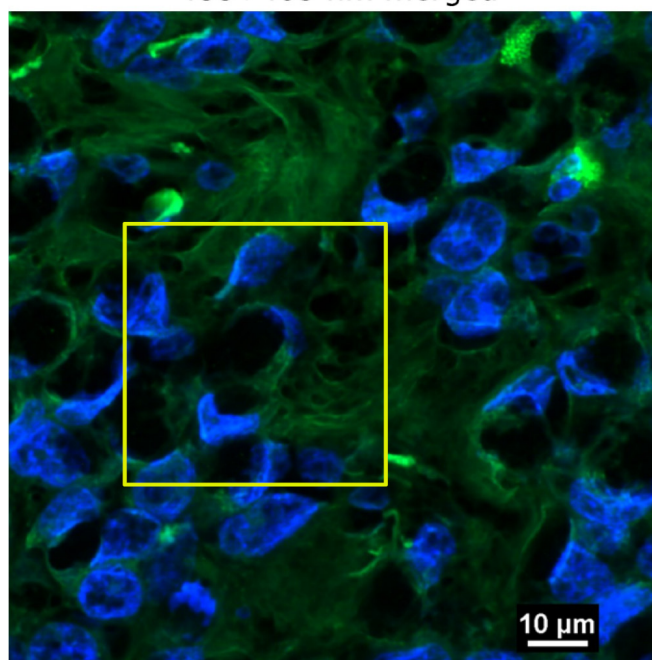


Figure 6. Colon signet ring cell carcinoma (SRCC) tissue

Alexa 488 labeled antibody stain for GPR 43 merged with DAPI-stained nuclei. Yellow square MFI (488 nm channel only) = 148.27.

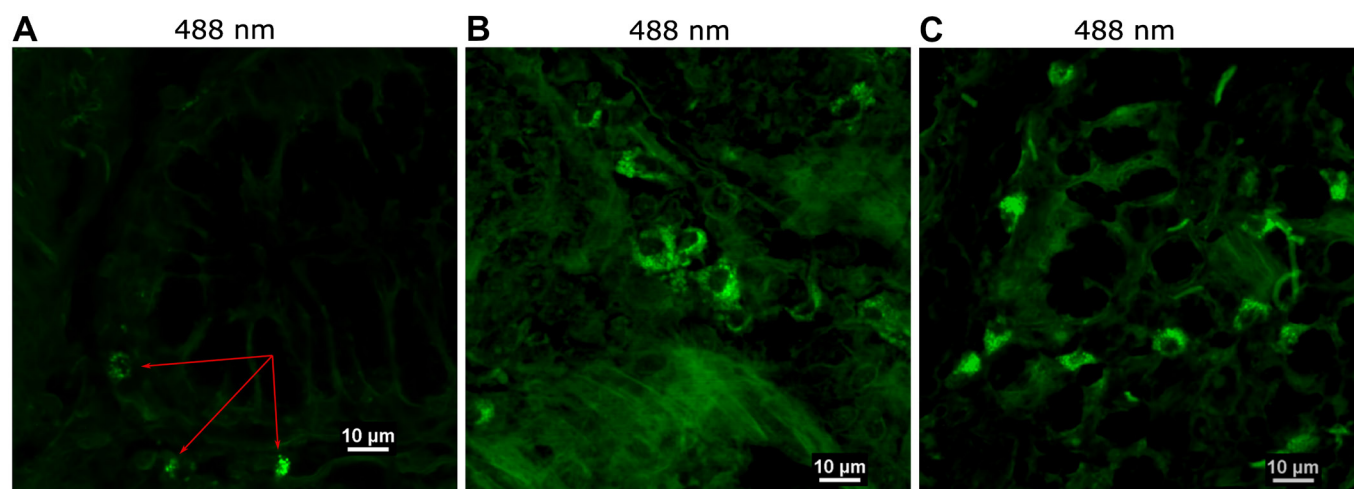


Figure 7. Eosinophils were imaged with the 488 nm diode laser

(A): Negative control from immunohistochemical staining (Note: red arrows point to Eosinophils). (B): Hematoxylin & eosin (H&E) stained sample. (C): Unstained sample.

DISCUSSION

The IHC staining protocol for GPR 43 appears to be effective on 3 μ m thick slices derived from formaldehyde-fixed, paraffin-embedded human colon tissue blocks. The negative controls presented no significant staining of tissues, with the only detected signal being attributed to auto-fluorescent structures (Eos) (Figures 3 and 7). The sample processing and staining protocol can be carried out on widely available formaldehyde-fixed, paraffin-embedded tissue samples, using commonly available, generic non-specialized reagents. The staining tray we designed, for which a printing model was provided free of intellectual property as part of the supplementary material, worked effectively to prevent the staining liquids from drying out and is readily manufacturable on most inexpensive, hobbyist-level additive manufacturing machines (3D printers). The staining procedure may also apply to other IHC staining epitopes, not only GPR 43.

While the presence of GPR 43 in human colonic tissue was previously reported [8,12] and quantitatively analyzed using classical IHC methods [14], the cellular level disposition was sparsely analyzed. The results from our study suggest that the majority of GPR 43 in colonocytes and goblet cells is concentrated at the apical and basal parts of the cells. This disposition would be explained by the fact that SCFAs, the main ligands for these receptors, are produced in the colon and then partially absorbed into the bloodstream [15]; thus, the colon lumen and the blood contain the highest concentrations of these receptors.

While healthy tissue presents a well organized and characteristic disposition of GPR 43 receptors, tumoral tissue, on the other hand, is much less organized, the dotted and relatively homogenous signal is likely to be a result of this. Nevertheless, tumoral tissue does appear to produce GPR 43 receptors, which leads to two possibilities. Firstly, it might have a similar function as in normal cells and is necessary for cells to function and divide. Secondly, while still being produced, due to the numerous mutations and general poor differentiation of tumoral tissue, it has no clear function and is expressed by the cells completely at random. The second hypothesis presents a higher probability due to the relatively disorganized disposition throughout the tissue. Tang et al. [14] have reported significantly lower staining of ACC tissue using classical IHC methods; however, this apparent reduction or complete lack of staining might be the result of ACC tissue expressing GPR 43 receptors diffusely and randomly, as presented in Figure 5. This erratic disposition is not apparent using classical IHC methods due to its inherent lower resolution, but can, however, be visualized with the aid of confocal microscopy. Further research is necessary to determine whether GPR 43 has any function in colon tumoral tissue. If this is confirmed, it could lead to novel treatments targeting these receptors.

In the case of SRCC, the tissue was relatively poorly stained, almost comparable to the negative control, whereas, on the same slide, healthy tissue sections presented similar signal intensity to the ones on the other IHC-stained specimens. There were areas with certain signals, however this was relatively heterogenous and without any specific disposition. SRCC is a relatively poorly differentiated type of CC [2,3]; therefore, there is a high probability that the cells do not produce GPR 43. Taking this into consideration, as well as the previously mentioned appearance of ACC tissue, increases the probability of the hypothesis that CC cells, due to the mutations suffered by neoplastic cells, aberrantly and randomly produce GPR 43, without having any specific function for the cell. If further research validates this hypothesis, then the amount of GPR 43 receptor produced by CC tissue may be directly proportional to the differentiation grade of the cells, thus being a possible criterion for evaluating the differentiation grade of neoplastic cells.

An additional finding was the strong autofluorescence of Eos present in the lamina propria as well as in multiple tumoral tissue areas. The autofluorescence of Eos was previously reported in the literature [16], and this property was also suggested as a possible method for the antibody-free separation of Eos via flow cytometry [17] as well as their detection using fluorescence lifetime imaging microscopy [18].

Jenvey and Stabel [19] attributed Eos autofluorescence to the presence of flavin dinucleotide (FAD), reduced nicotinamide-adenine dinucleotide (NADH), as well as reduced nicotinamide-adenine dinucleotide phosphate (NADPH), with FAD having a maximum emission between 500 and 560 nm, NADH between 445 and 520 nm, and NADPH between 440 and 470 nm. The maximum emission of NADH and NADPH either correspond or are extremely close to the emission peak of 490-510 nm, which characteristic of the fluorophore of the secondary antibody when excited at 488nm.

Blood Eos counts have been inversely correlated with the incidence of malignancies in general and CC in particular [20]. Peritumoral and intratumoral Eos counts in CC were significantly associated with better tumor differentiation and lower vascular invasion, while peritumoral Eos counts were an independent positive prognostic factor for survival rate [21]. The quantification of Eos in tumoral tissue samples thus has significant clinical value. The findings of our study present a novel positive microscopical identification method that could readily be carried out using classical fluorescence microscopy as well, even on unstained samples. The possibility of quickly quantifying the number and location (intratumoral versus peritumoral) of Eos in CC could lead to a better evaluation of the differentiation of CC tissue, leading to better therapeutic decisions, and being able to be used as a prognostic factor in disease evolution.

The two main limitations of the current study are the limited sample size and the lack of correlation of the identified receptors with other parameters, such as staining for GPR41 concomitantly or correlating with other immunohistochemistry markers commonly used in colorectal cancer diagnosis and staging, such as cytokeratin 20 and cytokeratin 7 [2]. Future studies that will be carried out on increased sample sizes would also enable the analysis of GPR43 in the context of different tumour localization and staging, since short-chain fatty acids concentrations vary along the colon from the cecum to the rectum [22].

Measuring MFI is inherently a semi-quantitative method for comparing images, due to the heterogeneity of microscopic images, with some having dead space with no tissue present. This leads to variations in the mean intensity of the signals. Future studies should focus on a more quantitative approach to measuring signal intensity; however, this still remains difficult to carry out on tissue samples, especially the colon, which presents a high number of goblet cells and crypts.

Although the present work was a pilot study that handled limited tissue samples, it focused on providing an effective immunohistochemistry staining method for GPR 43 on formaldehyde-fixed and paraffin-embedded tissue samples, using generic materials. It also analyzed the cellular level disposition of GPR 43 in human colon cancer, providing insights into its possible functions at the tumoral level, paving the way for future research in this area. If the short-chain fatty acids receptors play a crucial role in tumoral metabolism, they might present themselves as targets for future therapeutic agents in the treatment of colon cancer.

CONCLUSIONS

We found that the cellular level disposition of GPR 43 in healthy colon epithelial tissue is more concentrated at the apical and luminal membranes of colonocytes and goblet cells. Moreover, tumoral tissue produces GPR 43 in a random, disorganized, and heterogeneous fashion, which suggests that in the case of neoplastic tissue, GPR 43 might have an altered function. Eos present in healthy colon lamina propria, as well as peritumorally and intratumorally, exhibit a strong autofluorescence compared to surrounding tissue at 488 nm. Besides the cellular topic of IHC findings related to GPR 43 and Eos, the present study shows in detail an effective and easily reproducible IHC staining method for GPR 43 on formaldehyde-fixed and paraffin-embedded tissue samples, using only generic materials and bringing as an added value a 3D printing model for the IHC staining tray, free of intellectual property.

ABBREVIATIONS

Ac	– Acetate
ACC	– Adenocarcinoma
Bu	– Butyrate
CARB	– Citrate antigen recovery buffer
CC	– Colon cancer
DAPI	– 4',6-diamidino-2-phenylindole
DW	– Distilled water
Eos	– Eosinophils
FAD	– Flavin dinucleotide
FFAR 2	– Free fatty acid receptor 2
FFAR 3	– Free fatty acid receptor 3
GPR 41	– G-protein coupled receptor 41
GPR 43	– G-protein coupled receptor 43
H&E	– Haematoxylin and Eosin
IHC	– Immunohistochemistry
MFI	– Mean Fluorescence Intensity
NADH	– Nicotinamide-adenine dinucleotide
NADPH	– Nicotinamide-adenine dinucleotide phosphate
Prop	– Propionate
RCM	– Re-scanning confocal microscope
SCFAs	– Short-chain fatty acids
SRCC	– Signet ring cell carcinoma
TBS	– Tris-buffered saline
Tx	– Triton x-100 wash solution

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None.

AUTHORS' CONTRIBUTION

TI: conceptualization, methodology, validation, resources, data curation, writing (original draft)

ÉK: methodology, validation

MF: methodology, validation

AFZ: data curation, visualization

SRG: formal analysis, writing (review & editing), visualization

CNS: conceptualization, writing (review & editing)

AMC: conceptualization, formal analysis, supervision, project administration

All authors have read and approved the final version of the manuscript.

CONFLICT OF INTEREST

None to declare.

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