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Basic Study

Roles of phosphatidylinositol-3-kinases signaling pathway in inflammation-related cancer: Impact of rs10889677 variant and buparlisib in colitis-associated cancer

Nurul Nadirah Razali, Raja Affendi Raja Ali, Khairul Najmi Muhammad Nawawi, Azyani Yahaya, Norshafila Diana Mohd Rathi, Norfilza Mohd Mokhtar

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Nurul Nadirah Razali, Norshafila Diana Mohd Rathi, Norfilza Mohd Mokhtar, Department of Physiology, Faculty of Medicine, Universiti Kebangsaan Malaysia, Cheras 56000, Kuala Lumpur, Malaysia

Raja Affendi Raja Ali, School of Medical and Life Sciences, Sunway University, Sunway City 47500, Malaysia

Raja Affendi Raja Ali, Khairul Najmi Muhammad Nawawi, Norfilza Mohd Mokhtar, GUT Research Group, Faculty of Medicine, Universiti Kebangsaan Malaysia, Cheras 56000, Kuala Lumpur, Malaysia

Raja Affendi Raja Ali, Khairul Najmi Muhammad Nawawi, Gastroenterology Unit, Department of Medicine, Faculty of Medicine, Universiti Kebangsaan Malaysia, Cheras 56000, Kuala Lumpur, Malaysia

Azyani Yahaya, Department of Pathology, Faculty of Medicine, Universiti Kebangsaan Malaysia, Cheras 56000, Kuala Lumpur, Malaysia

Corresponding author: Norfilza Mohd Mokhtar, PhD, Professor, Department of Physiology, Faculty of Medicine, Universiti Kebangsaan Malaysia, Jalan Yaacob Latif, Bandar Tun Razak, Cheras 56000, Kuala Lumpur, Malaysia. norfilza@ppukm.ukm.edu.my

Abstract

BACKGROUND

Phosphatidylinositol-3-kinases (PI3K) is a well-known route in inflammation-related cancer. Recent discovery on PI3K-related genes revealed a potential variant that links ulcerative colitis (UC) and colorectal cancer (CRC) with colitis-associated cancer (CAC). PI3K/AKT pathway has been recommended as a potential additional therapeutic option for CRC due to its substantial role in modifying cellular processes. Buparlisib is a pan-class I PI3K inhibitor previously shown to reduce tumor growth.

AIM

To investigate the regulation of rs10889677 and the role of buparlisib in the PI3K signaling pathway in CAC pathogenesis.

METHODS

Genomic DNA from 32 colonic samples, including CAC ($n = 7$), UC ($n = 10$) and CRC ($n = 15$), was sequenced for the rs10889677 mutation. The mutant and wildtype fragments were amplified and cloned in the pmirGLO vector. The luciferase activity of cloned vectors was assessed after transfection into the HT29 cell line. CAC mice were induced by a mixture of a single azoxymethane injection and three cycles of dextran sulphate sodium, then buparlisib was administered after 14 d. The excised colon was subjected to immunohistochemistry for Ki67 and Cleaved-caspase-3 markers and quantitative real-time polymerase chain reaction analysis for *Pdk1* and *Sgk2*.

RESULTS

Luciferase activity decreased by 2.07-fold in the rs10889677 mutant, confirming the hypothesis that the variant disrupted miRNA binding sites, which led to an increase in *IL23R* expression and the activation of the PI3K signaling pathway. Furthermore, CAC-induced mice had a significantly higher disease activity index ($P < 0.05$). Buparlisib treatment significantly decreased mean weight loss in CAC-induced mice ($P < 0.05$), reduced the percentage of proliferating cells by 5%, and increased the number of apoptotic cells. The treatment also caused a downward trend of *Pdk1* expression and significantly decreased *Sgk2* expression.

CONCLUSION

Our findings suggested that the rs10889677 variant as a critical initiator of the PI3K signaling pathway, and buparlisib had the ability to prevent PI3K-non-AKT activation in the pathophysiology of CAC.

Key Words: Colitis-associated cancer; Colorectal cancer; Phosphatidylinositol 3-kinase; Animal model; Luciferases; Renilla; Phosphatidylinositol 3-kinase inhibitor

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Core Tip: The role of phosphatidylinositol-3-kinases (PI3K) in promoting cancer progression has been widely acknowledged due to its crucial involvement in regulating the survival, differentiation, and proliferation of cancer cells. Here, we investigate the role of PI3K signaling in colitis-associated cancer (CAC) pathogenesis by studying the regulation of potential variant in PI3K-related gene in the colorectal cancer cell line and the utilization of PI3K inhibitor, buparlisib, in the CAC-induced mice model. We suggested that rs10889677 variant plays a crucial role in initiating the PI3K signaling pathway, and buparlisib has the capability to inhibit PI3K-non-AKT activation in the pathophysiology of CAC.

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INTRODUCTION

Inflammatory bowel disease (IBD), a chronic inflammatory disorder that goes into remission and then recurs, eventually leading to the development of colitis-associated cancer (CAC), is a kind of colorectal cancer (CRC)[1,2]. Crohn's disease and ulcerative colitis (UC), the two main subtypes of IBD, had CAC risks of 1.4% and 0.8%, respectively[3,4]. The rising trend of IBD incidence and prevalence among Asians increased from 1.3% to 7.2% per 100000 population among Malaysian-Singaporeans[5]. Rapid urbanization, adoption of a Western diet, antibiotics use, personal hygiene standards, microbiological exposures, and pollution are all risk factors for developing IBD[6]. In Malaysia, the average annual incidence of IBD has risen to 1.46 per 100000 people over the last decade[7]. CAC accounted for 10%-15% of IBD fatality cases among Westerners, accounting for only 1% to 2% of CRC incidence in Malaysia[4,8].

CAC develops through the inflammation-dysplasia-carcinoma pathway[9]. Immune cells, including cytokines, chemokines, epithelial cells, and stromal cells, are among the cell types contributing to the inflammatory processes in the CAC development[10]. Moreover, inflammatory mediators had a substantial impact on the control of pre-neoplastic growth during CAC development, and their release is more likely to target multiple carcinogenesis-related signaling pathways involved, such as NFκB, PI3K, JAK/STAT and Wnt/B-catenin[11,12].

Phosphatidylinositol-3-kinases (PI3K) signaling pathway is an essential intracellular signaling mechanism in regulating the cell cycle[13]. Growth factors, cytokines, and other stimulatory chemicals activate *PI3K* via their receptors. The *AKT-mTOR* and *SGK* families receive signals from activated *PI3K*, which controls cell growth, proliferation, and apoptosis[14]. *PI3K* enzymatic activity has been linked to the aetiology of various diseases, including chronic inflammation and cancer [12,15]. Our earlier research identified *PI3K* as one of the critical pathways in long-duration UC associated with an increased risk of developing CAC[16]. A recent study on mutational analysis in CAC patients identified several potential

variants, including rs10889677, in the *IL23R*, a cytokine-induced PI3K-related gene that may link inflammation to an increased risk of cancer[17].

PI3K/AKT pathway has been proposed as a viable therapeutic target for CRC due to its significant role in modulating cellular processes by targeting the downstream pathway[18]. Buparlisib is an oral pan-class I PI3K inhibitor that targets all four class I PI3K catalytic isoforms (p110 α , p110 β , p110 δ and p110 γ)[19,20]. This drug is the most clinically advanced medication to have passed a phase II clinical trial in the cancer[21,22]. Buparlisib treatment in *in vivo* study showed encouraging outcomes by enormously lowering the quantity of phosphorylated AKT and slowing tumor growth in tumor-bearing mouse models[19,23]. Therefore, the objective of this study was to study the regulation of the PI3K-related variant, rs10889677, in the CRC cell line and to investigate whether buparlisib may affect the non-AKT-independent branch of the PI3K signaling pathway, which was one of the keys signaling pathways implicated in cancer development and progression, using a mouse model.

MATERIALS AND METHODS

Sample collection and preparation

The Universiti Kebangsaan Malaysia Research Ethics Committee (UKM/PPI/111/8/JEP-2019-572) approved this study. A total of 32 fresh frozen and archival samples (ranging from the year 2014 to 2019) from patients with long-standing UC, CAC, and CRC were collected from the Endoscopy Unit at Universiti Kebangsaan Malaysia Medical Centre, Kuala Lumpur, Malaysia. Upon admission, all patients provided informed consent. All tissues were collected by December 2020, stored in RNAlater, and frozen at -80 °C until further processing. The hematoxylin and eosin-stained tissue sections were evaluated by an experienced pathologist for confirmation of diagnosis, inflammation, and metastatic status. The genomic DNA was extracted from fresh frozen tissues using the AllPrep DNA/RNA/miRNA Universal Kit (Qiagen, United States) according to the manufacturer's protocol. Meanwhile, GENEREAD DNA FFPE Kit (Qiagen, United States) was used to extract DNA from formalin-fixed paraffin-embedded blocks of archival samples. DNA concentration was measured using DeNovix DS11+ Spectrophotometer (DeNovix Inc., United States).

Sanger sequencing

Primers were designed using the NCBI Primer Tool (National Center for Biotechnology Information, United States) and Primer3Plus[24] based on the location of the somatic variant. The predesigned primers were as follows: *IL23R*, forward 5'-TCT GTG CTC CTA CCA TCA CC-3', and reverse 5'-TGT GCC TGT ATG TGT GAC CA-3'. SnapGene Viewer 5.3.2 was used to analyze the sequencing results.

Fragment amplification

The fragments containing the wildtype and mutant rs10889677 variant on the 3' untranslated region of *IL23R* were amplified from the genotyped DNA samples. The predesigned primers were added with the target sequences of the *XbaI* and *XhoI* restriction enzymes. The primers were as follows: Forward 5'-ATC GCT CGA GGC TGC CTT GCA ATC TGA ACT-3' and reverse 5'-ATC GTC TAG ATC TGC CTT CCT GGT TCA AGT-3'. The polymerase chain reaction for fragment amplification was carried out using GoTaq® Green Master Mix (Promega, United States) with a pre-denaturation step at 95 °C for eight minutes, followed by 35-40 cycles of 95 °C for 30 s, 60 °C for 30 s and 72 °C for 30 s, with an additional final elongation step at 72 °C for five minutes. Then, the polymerase chain reaction (PCR) products were run on gel electrophoresis to check for fragment bands, followed by the gel purification step using the QIAquick® Gel Extraction Kit (Qiagen, United States).

Plasmid preparation and cloning

A total of 1 μ g of pmirGLO (Promega, United States) vector was digested with the following enzymes: *XbaI* (NEB, United Kingdom) and *XhoI* (NEB, United Kingdom) according to the manufacturer's instructions. The ligation reactions were prepared using a T4 DNA Ligase kit (NEB, United Kingdom) with a molar ratio of 1:3 vector to insert. The ligated products were transformed utilizing the heat-shock technique with an *E.coli* competent cell (NEB, United Kingdom). Colonies from prior successful transformations were screened, and positive transformant plasmid was purified using PureYield™ Plasmid Miniprep System (Promega, United States).

Transfection and luciferase assay

The CRC cell line, HT29, was routinely cultured in a RPMI-1640 (Elabscience, United States) supplemented with 10% fetal bovine serum (Gibco, United States) and 1% of Penicillin/Streptomycin (Gibco, United States) and cultivated in 5% of CO₂ level incubator at 37 °C. Before transfection, 5.0 $\times 10^4$ HT29 cells were seeded into a 96-well white plate in 100 μ L antibiotic-free growth media. Cultures were performed in triplicate. A total of 0.25 μ g of wildtype, mutant and empty plasmids were transfected using Lipofectamine 2000 (Invitrogen, United States) transfection reagent according to the manufacturer's protocol. Afterwards, the luciferase activity was measured 48 h after post-transfection using the Dual-Glo® Luciferase Assay system (Promega, United States) following the manufacturer's protocol. Firefly and Renilla luciferase activity were measured using the GloMax® 20/20 Luminometer (Promega, United States). The assay was performed in triplicate, and all results were normalized with the internal control reading.

Animals

Forty male Balb/c mice (seven to eight weeks old, weighing 25-30 g) were purchased from the Animal Unit, Faculty of Science and Technology, Universiti Kebangsaan Malaysia, Bangi, Malaysia. The mice were housed in individual cages with kenaf bedding and maintained on a 12 h light/dark cycle with controlled humidity at 25 °C kept in the animal laboratory unit, Faculty of Medicine, Universiti Kebangsaan Malaysia. Regular food and water were supplied *ad libitum*. All mice were acclimatized for ten days before the start of the treatment. The animal handling and protocol was approved by the Animal Ethics Committee of Universiti Kebangsaan Malaysia (PPUKM/2019/NORFILZA/25-SEPT./1035-SEPT.-2019-DEC.-2021).

Chemical induction and PI3K inhibitor administration

The mice were randomly divided into four groups ($n = 10$ each): Control, dextran sodium sulphate (DSS) induced, azoxymethane (AOM) induced, and AOM/DSS combination induced[25]. A single intraperitoneal injection of AOM (10 mg/kg body weight) (Sigma-Aldrich Chemicals, United States) was administered to the AOM and AOM/DSS-induced group. A week after injection, AOM/DSS and DSS-induced groups received 2.5% DSS (40kDa; Sigma-Aldrich Chemicals, United States) in their drinking water for seven days in three cycles (each cycle consisting of one-week DSS, followed by two weeks of sterile water). The control and AOM-induced groups were only provided with sterile water throughout the treatment. Beginning in week eight, mice from the AOM/DSS-induced group were treated daily for 14 d with a PI3K inhibitor buparlisib (NVP-BKM120, MedChemExpress, United States) at 30 mg/kg *via* oral administration (Figure 1).

Disease activity index scoring

Daily observations and records were made of body weight, bowel consistency, and rectal bleeding throughout ten weeks. Changes in these factors will be scored to determine the disease activity index (DAI), which is based on the rating of each component. Scores were assigned for weight loss (0: 0-1%, 1: 1%-5%, 2: 5%-10%, 3: 10%-20%, 4: > 20%), stool consistency (0: Normal, 2: Loose stool, 4: Diarrhea), and presence of blood in the stool (0: Negative, 2: Visual blood in stool, 4: Fresh rectal bleeding)[26]. A total score was determined, ranging from 0 to 12.

Histological examination

Colons were removed, cleaned, and flushed with sterile phosphate buffer saline and cut into two parts: Proximal and distal colon. The proximal colon was stored in RNAlater solution (ThermoFisher, United States), and the distal colons were rolled, placed in the tissue cassette, and soaked in 10% neutral-buffered formalin for at least one night. Then, the tissues were processed, embedded in paraffin, and sectioned for hematoxylin and eosin staining. Histological structures were assessed and scored (0: Healthy colon, 1: Minimal inflammation with minimal to no separation of crypts, 2: Mild inflammation with mild separation of crypts, 3: Moderate inflammation with separation of crypts, with or without focal effacement of crypts, 4: Extensive inflammation with marked separation and effacement of crypts, 5: Diffuse inflammation with marked separation and effacement of crypts)[27]. The pathologist used an inverted microscope to examine all slides.

Immunohistochemistry

Immunohistochemistry (IHC) staining was performed using the Rabbit-specific HRP/DAB Detection IHC Detection kit Micro-polymer, as instructed by the manufacturer (ab236469; Abcam, United States). Slides were incubated in primary antibodies: Rabbit monoclonal Cleaved caspase-3 (CC-3) (1:5000, ab214430; Abcam, United Kingdom) and rabbit monoclonal Ki67 (1:100, ab16667; Abcam, United Kingdom) for 30 minutes. The secondary antibody was goat anti-rabbit HRP Conjugate, included in the same kit. Images were obtained using an Olympus light microscope (Japan). Qualitative and quantitative scoring were scored blindly by the experienced pathologist.

Quantitative real-time PCR

The excised proximal colon was sectioned into smaller fragments and placed in different tubes at -80 °C. RNA was prepared using AllPrep DNA/RNA/miRNA Universal Kit (Qiagen, United States) following the manufacturer's instructions. The total RNA yield was quantified using a DS-11 spectrophotometer (DeNovix Inc., United States). cDNA conversion was conducted using QuantiNova Reverse Transcription Kit (Qiagen, United States) from 1 µg RNA following the manufacturer's instructions. Quantitative PCR amplifications were prepared with approximately 100 ng cDNA using the QuantiNova SYBR Green PCR Kit (Qiagen, United States) and mouse primer sets (IDT, United States). The predesigned primers were as follows: *Ppia*, forward 5'-CAA ACA CAA ACG GTT AG-3' and reverse 5'-TTC ACC TTC CCA AAG ACC AC-3'; *Sgk2*, forward, 5'-GCA TAG AGC CTA CCT GAT CAC-3' and reverse 5'-CCC AGG TTG ATG TTC CCA TT-3' and *Pdk1*, forward 5'-CAT GCA CAT CCA GAT CAC AGA-3' and reverse 5'-CTT TTA CAC GCC GAC TTC TCT-3'. The gene expression was normalized to the reference gene, *Ppia* and all reactions were prepared in triplicate.

Statistical analysis

Normally distributed variables are presented as the mean $\pm$ standard error mean. For statistical significance, the Student T-test and ANOVA tests were performed. At $P < 0.05$, the differences between groups were considered significant. Statistical analyses were performed using the GraphPad Prism Version 9.3.1 (GraphPad Software Inc., United States).

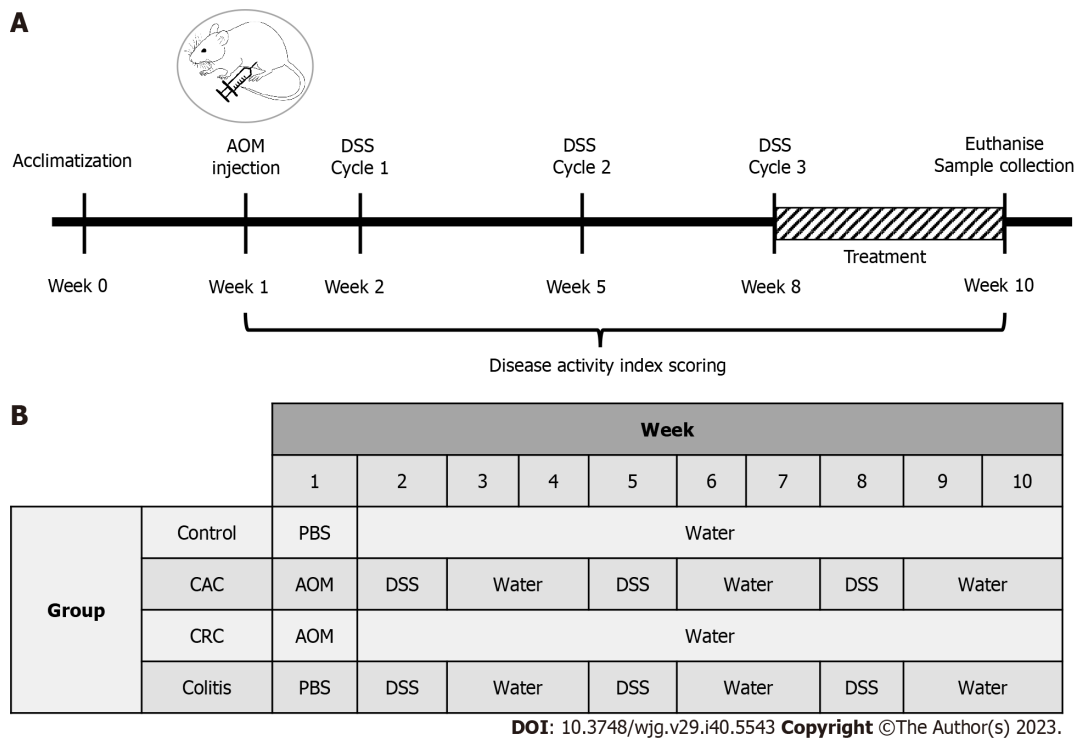


Figure 1 Animal induction procedure. A: The experimental design for the chemical induction procedure; B: The schematic diagram of mice model grouping. DSS: Dextran sodium sulphate; AOM: Azoxymethane; CAC: Colitis-associated cancer; CRC: Colorectal cancer.

RESULTS

Information in clinical samples

Table 1 displays demographic data for all samples. The median age of all samples was 63 years old (IQR:11.5). Malays made up the majority of the samples (56%) and were followed by Chinese (28%) and Indians (15%). The gender distribution was equal between males and females. Most patients' smoking status was non-smoker (91%) instead of smoker/ex-smoker (9%). Most UC patients were diagnosed with left-sided colitis or pancolitis, with a Mayo index score of 1 to 3 and a Geboes score of Grade 2A.1 to 2A.2. The majority of CRC and CAC patients were at stages 1 and 3, and the rectosigmoid and distal colon were the sites of the malignancies. Histologically, most CRCs were moderately differentiated, whereas tumors from CAC patients were categorized as poor, moderate and well differentiated. There was no reported familial history of CRC among any of the patients.

PI3K-related somatic variant rs10889677 exhibited a reduced trend of luciferase activity in CRC cell line

To understand the significance of the PI3K signaling pathway in the formation of CAC, the functional role of PI3K-related somatic variants that potentially correlate CAC with UC and CRC was investigated to gain insight into the role. Somatic variant rs10889677C>A has been successfully sequenced in CAC, UC and CRC samples. The proportion of homozygous CC (wildtype) was discovered to be the highest in CRC samples (60%), while the proportion of homozygous AA (mutant) was highest in CAC samples (85%). We discovered mutant rs10889677 had a 2.07-fold lower luciferase activity (2.35 ± 0.85) than the wildtype construct (4.42 ± 0.86). However, the difference was not statistically significant (**Figure 2**).

Combination of AOM + DSS produced a successful CAC-induced mice model

In a CAC mouse model, chemical induction of a single AOM injection with three cycles of 2.5% DSS was considered successful. The assessment of a successful CAC induction was based on observing body weight loss, the condition of the stool and the occurrence of any rectal bleeding. The CAC mice group endured reduced body weight, watery stools, and rectal bleeding for ten weeks. In light of this, the CAC mice group scored significantly higher on the DAI than the colitis and control groups, with a mean score of 1.25 ± 0.22 ($P < 0.05$) (**Figure 3A**). The DAI score of the CAC mice group began to rise dramatically after the second DSS induction (**Figure 3B**). On the other hand, the colitis group had a low DAI score, while the control group had none.

Buparlisib therapy was able to reverse the weight loss in the CAC-induced mice model

Due to ongoing DSS exposure, the colitis and CAC-induced mice group's body weights have fluctuated (**Figure 4**). At every DSS induction cycle, weight loss was predicted to occur. Following each cycle, mice had a two-weeks recovery period to help them regain any lost weight. However, starting with the third cycle of DSS induction, the CAC mice group had substantial weight loss, losing about 5% of their total body weight. This significant body weight reduction in the CAC group explained why they had a higher DAI score than other groups. Meanwhile, the mice in the other groups

Table 1 Clinical and demographic details of the recruited patients

	CRC (n = 15)	CAC (n = 7)	UC (n = 10)
Median age (range)	63 (39-67)	58 (20-71)	65 (52-69)
Race			
Malay	11	2	5
Chinese	4	2	3
Indian	-	3	2
Gender			
Male	9	3	4
Female	6	4	6
Smoking status			
Smoker/Ex-smoker	2	1	-
Non-smoker	13	6	10
Stage ¹			
I	2	1	Not applicable
II	-	-	
III	7	4	
Adenocarcinoma types ¹			
Poorly differentiated	-	1	Not applicable
Moderately differentiated	7	1	
Well differentiated	1	3	
Mayo score (range) ¹	Not applicable	Data unavailable	1-3
Geboes score (range) ¹	Not applicable	Data unavailable	2A.1-2A.2
Presentation	Rectal bleeding	Rectal bleeding, abdominal pain, severe diarrhea	Diarrhea, loss of appetite

¹Some data are unavailable.

All data are expressed as n except where indicated in the table. UC: Ulcerative colitis; CRC: Colorectal cancer; CAC: Colitis-associated cancer.

recovered and kept their body weight throughout the treatment (Figure 4A).

PI3K inhibitor (buparlisib) supplementation, started in the eight week of treatment, was used to perform additional research on the relationship between PI3K and the advancement of CAC. PI3K oral inhibitor dosage was calculated depending on the body weight of CAC mice. After receiving daily treatment for 14 d, the mean weight loss (g) in the CAC-induced mice was significantly lower (2.0 ± 0.0) than in the CAC group that had not received any treatment (2.6 ± 1.07) ($P < 0.05$) (Figure 4B).

CAC-induced animals exhibited inflammation and tumor growth characteristics

The distal colon underwent histological evaluation to check for any morphological alterations, and the results were reported in Figure 5. No diffuse inflammation was observed in any of the tissues in the present study, which ranged from 0 (no inflammation/healthy), 1 (minimal inflammation), 2 (mild inflammation), 3 (moderate inflammation), and 4 (extensive inflammation). Most control group mice had healthy colons with uniformly shaped glands and minimal inflammation. In the colon tissues of the colitis mice group, mild to moderate inflammation with separation of crypts, with or without focal effacement of crypts, and various gland shapes were seen. On the other hand, at least 50% of CAC-induced mice had tumors and varying degrees of mild to severe inflammation. These tumors had enlarged nuclei, reactive cellular changes, moderate inflammatory response, depleted glands, and an elevated neutrophil count. The scoring on colitis and CAC mice colon tissue were scored depending on the inflamed area (chronic) or specifically at the tumor location (Figure 6).

Buparlisib treatment had anti-proliferative and pro-apoptotic actions in the CAC-induced mice model

The apoptotic marker (CC-3) and the proliferative marker (Ki67) were both subjected (Figure 7). Every type of tissue, even the healthy tissue, had proliferating cells. The proliferative cells' distribution might vary depending on the different tissue types. In the control group, the healthy colon exhibited 30% of Ki67-positive cells, with the proliferating cells predominating in the basal regions of the glands. In contrast, more than 50% and 80% of Ki67-positive cells had irregular

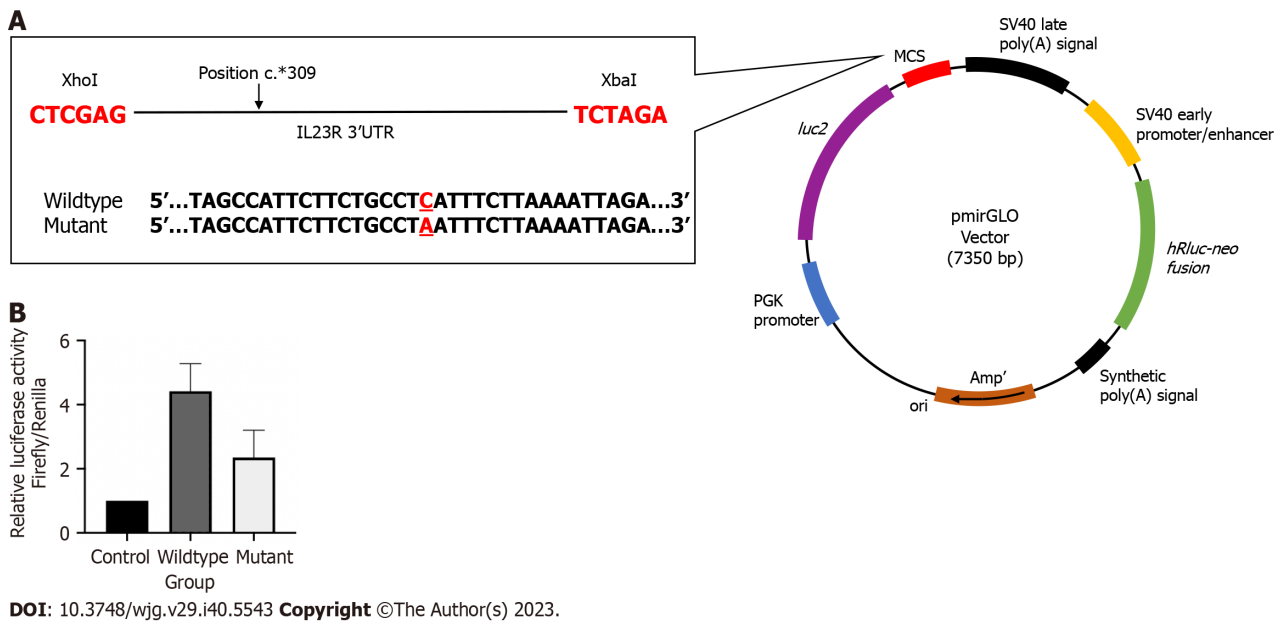


Figure 2 Dual-luciferase reporter assay for rs10889677 construct. A: The structure of luciferase vector, pmirGLO, with wildtype and mutant constructs; B: Luciferase activity of HT29 cell line transfected with a construct containing wildtype and mutant variant of rs10889677. Data are presented as mean $\pm$ SEM; one-way ANOVA was employed; $n = 3$.

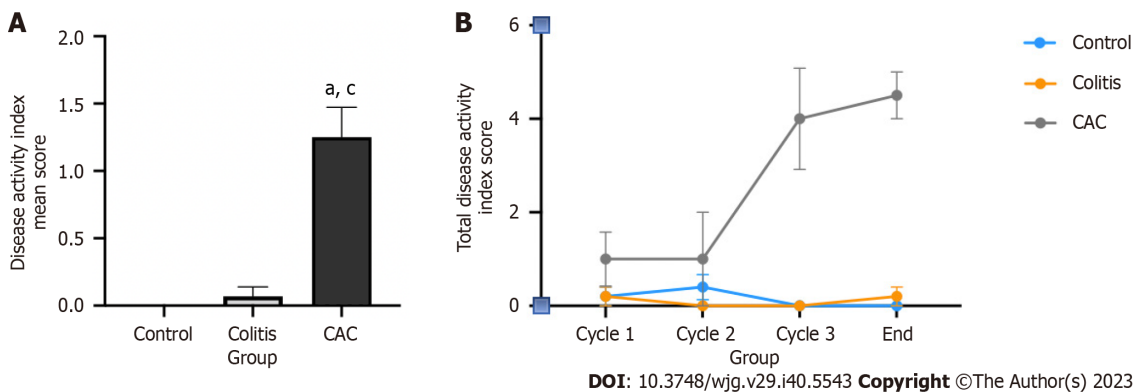


Figure 3 The disease activity index score. A: The total average disease activity index (DAI) score per group at the end of treatment. $^aP < 0.05$ (compared to the control group); $^cP < 0.05$ (compared to the colitis group); data are presented as mean $\pm$ SEM; one-way ANOVA was used; $n = 8$; B: The total average DAI score per group at each dextran sodium sulphate-induction cycle. Two-way ANOVA was used. CAC: Colitis-associated cancer.

distribution in the colitis and CAC mice groups, respectively. The uneven distribution of proliferative cells, particularly in the upper section of the glands, suggested the cells were highly proliferating. Buparlisib administration to the CAC mice group reduced about 5% of the positive cells compared to the untreated CAC mice group (Figure 7).

CC-3 was assessed as an apoptotic marker based on its intensity and the number of positive cells. Most tissues from the control, colitis and CAC mice group have moderate intensity of CC-3. In contrast to the control group, which had fewer than 50% positive cells, most colitis and CAC mice had more than 75% CC-3 positive cells. Based on the PI3K inhibitor treatment evaluation, buparlisib-treated CAC mice achieved a high overall score of CC3-intensity and -positive cells (6/8) (Figure 7).

Buparlisib was successful in reducing the activity of PI3K-non-AKT-associated genes

The relative gene expression of the downstream PI3K-non-AKT pathway genes *Pdk1* and *Sgk2*, which are involved in CAC progression, was assessed to study further the impact of the PI3K inhibitor on CAC development. Buparlisib treatment showed a downward trend in the expression of *Pdk1* (2.04 ± 1.17) in the CAC-induced mice group, however, it is not significant compared to the untreated group (Figure 8). Only *Sgk2* expression showed a significant reduction in treated CAC-induced mice (2.56 ± 1.09) in comparison to the untreated CAC mice (5.61 ± 0.79) ($P < 0.05$).

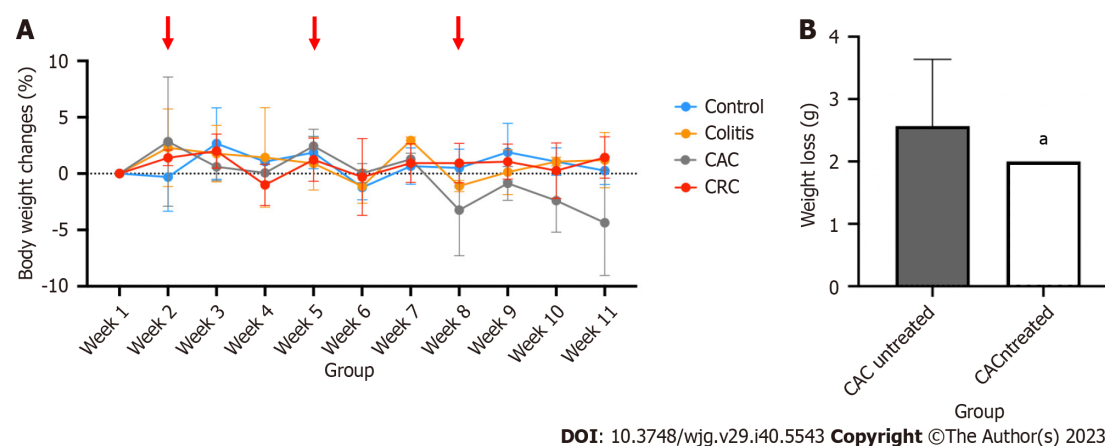


Figure 4 The body weight changes. A: Percentage of weight loss (%) in all groups. A drop in body weight was seen in the colitis-associated cancer (CAC) group after every cycle of dextran sodium sulphate (DSS) induction. Red arrows indicate the cycle of DSS induction. Two-way ANOVA was employed; $n = 8$; B: Total mean body weight loss (g) between buparlisib-treated and -untreated CAC-induced mice group. ^a $P < 0.05$ (compared to the CAC untreated group); data are presented as mean $\pm$ SEM; student T-test was employed; $n = 4$. CAC: Colitis-associated cancer; CRC: Colorectal cancer.

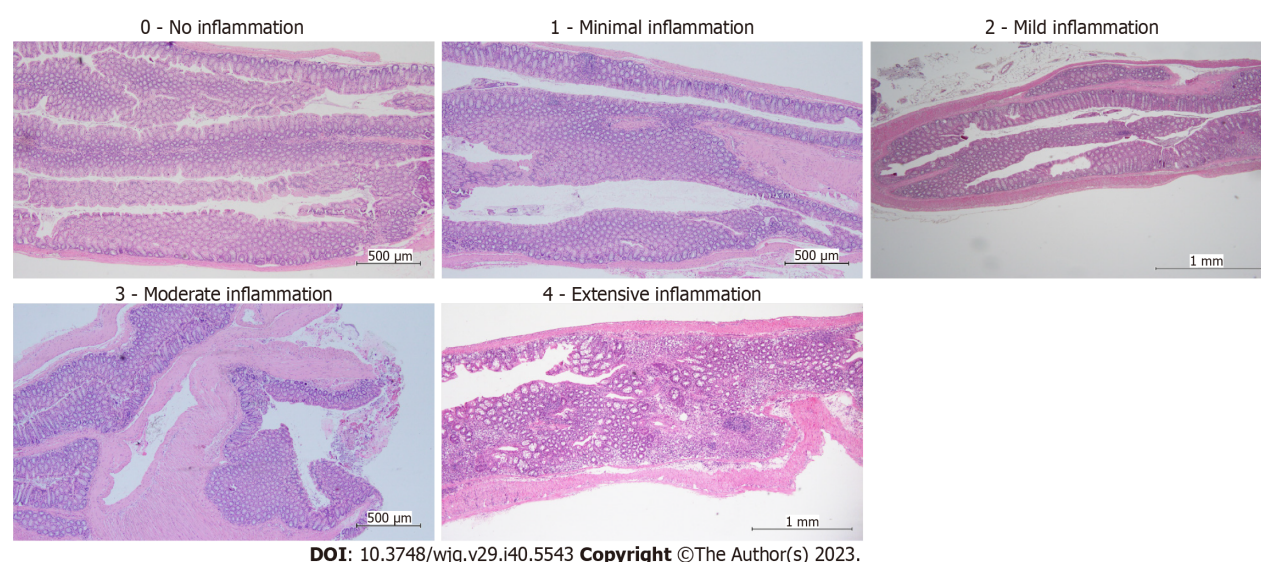
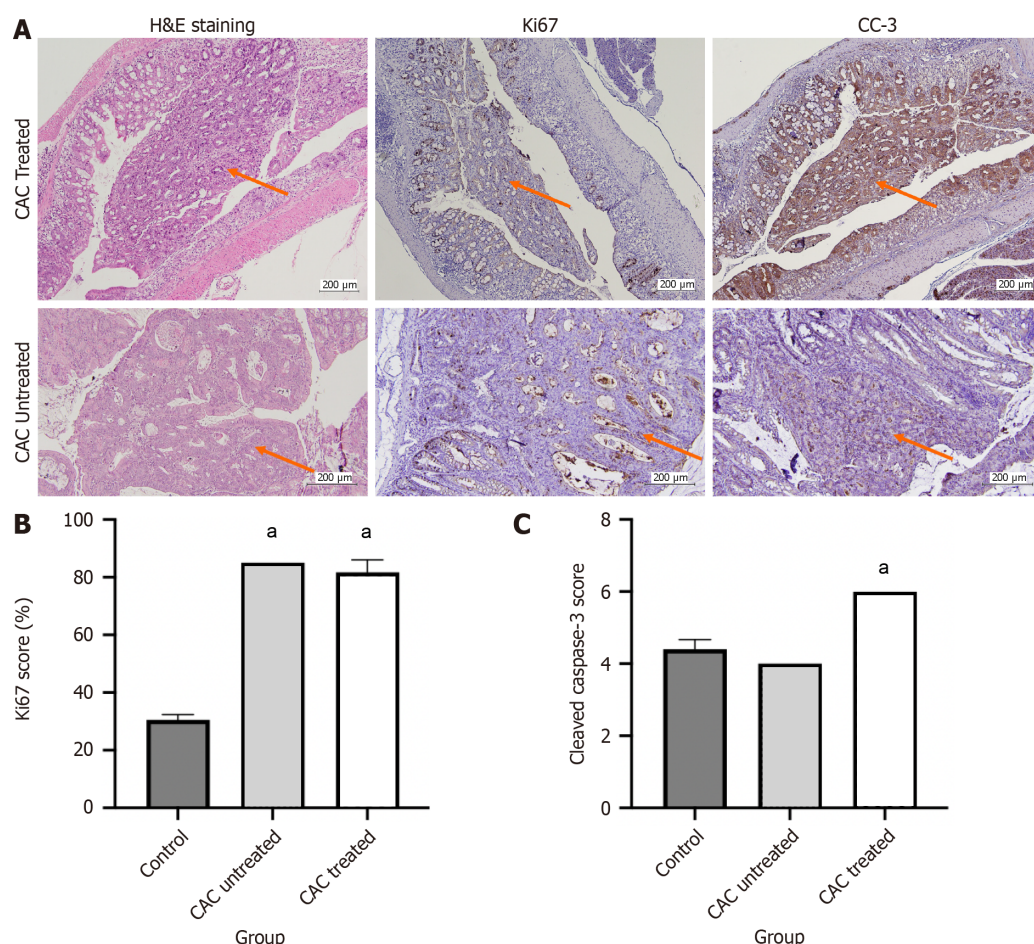


Figure 5 Histological structure assessment. The range of the histologic scores varied from 0 (no inflammation) to 4 (extensive inflammation). Magnification is 40 $\times$.



Figure 6 Histological examination of the control, colitis and colitis-associated cancer mice model. The control group exhibited a healthy colon with a uniform gland shape. Meanwhile, the colitis mice group showed mild to moderate inflammation. The tumor was only found in the colitis-associated cancer-induced mice group. Red arrows indicate the inflamed area and tumor sites. Magnification is 100 $\times$.



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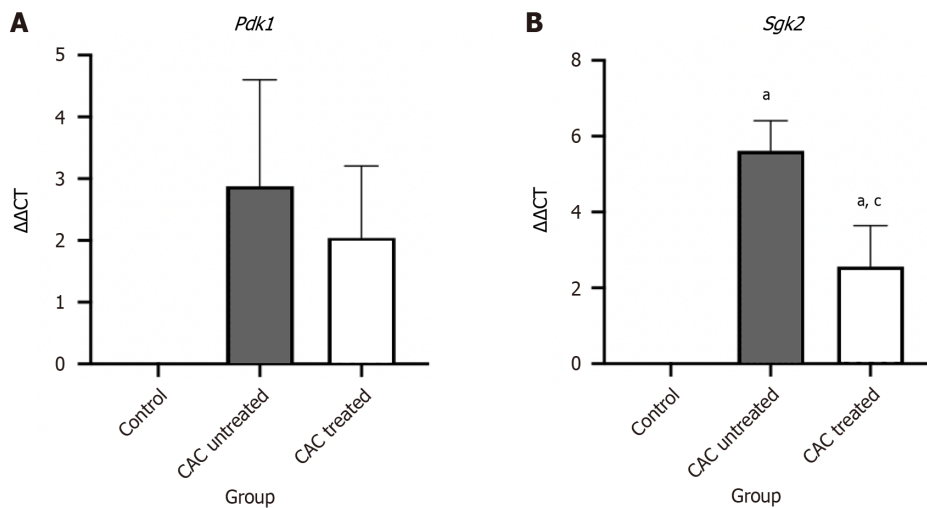
Figure 7 Immunohistochemistry analysis on Ki67 and cleaved caspase-3 markers on colitis-associated cancer-mice model. A: H&E staining, Ki67 and cleaved caspase-3 (CC-3) staining; B and C: Ki67 and CC-3 staining exhibited lower proliferative and higher apoptotic cells on the buparlisib-treated colitis-associated cancer (CAC) mice model compared to the untreated CAC mice. ^a $P < 0.05$ (compared to the control group); data are presented as mean $\pm$ SEM; one-way ANOVA was employed; $n = 4-8$. The red arrow showed the tumor area. Magnification is 100 $\times$. CAC: Colitis-associated cancer; CC-3: Cleaved caspase-3.

DISCUSSION

A well-known pathway in the emergence of inflammation-related cancer is the PI3K pathway. Dysregulation of the PI3K signaling pathway may affect cellular development, metabolism, proliferation, and apoptosis[28]. Recent research on the function of cytokine-induced PI3K-related genes in CAC patients led to the discovery of possible mutations that might connect CAC with UC and CRC[17]. We are, therefore, interested in learning more about the pathophysiology of CAC and how the PI3K signaling pathway is involved. In this study, we utilized dual luciferase assay to investigate the functional significance of rs10889677, a somatic mutation related to PI3K, and we generated CAC in mice to assess the function and regulation of PI3K inhibitor in the modulation of PI3K-non-AKT signaling pathway.

The single nucleotide polymorphism (SNP) rs10889677 is a genetic variant identified in the *IL23R*, one of the cytokine-induced PI3K-related genes. This particular SNP is a variation in the DNA sequence of the *IL23R*, where a single nucleotide (cytosine, C) is replaced by another nucleotide (adenine, A). The rs10889677 SNP is situated precisely in the 3' untranslated region of *IL23R*, which is also a miRNA binding site[29]. Any SNPs present in the miRNA binding site may affect how miRNA-mRNA interacts, changing the stability or translation efficiency of the mRNA[30]. This, in turn, may influence the expression of the *IL23R* protein, which is involved in immune response and inflammation, and may increase the susceptibility to certain diseases, such as CRC. The rs10889677 mutation has been linked strongly to IBD and several other cases, including breast, lung, bladder and colorectal[31-33]. In our recent publication, we have successfully predicted a notable decrease in the stability of mRNA secondary structure for the rs10889677 variant construct compared to the wildtype[17]. This finding supported the hypothesis that genetic mutations resulted in mRNA structural instability and disrupted the binding sites[17].

About 60%-70% of human CRC s were associated with activating the PI3K pathway, increased *AKT* signaling and *PTEN* inactivation[34]. Furthermore, PI3K inhibitors have been proposed as another effective targeted therapy for solid tumors, including CRC[35,36]. Nevertheless, further elucidation regarding the utilization of PI3K inhibitors in the context of CAC is warranted. Balb/c male mice were chosen because they are more susceptible and prone to grow tumors than other strains, and tumors were induced in them with a combination of AOM injection and repeated DSS cycles[37,38]. The application of combined AOM and DSS is a potent, reproducible, and reasonably priced initiation-promotion



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Figure 8 The relative gene expression for *Pdk1* and *Sgk2*. A: The relative gene expression of *Pdk1* in colitis-associated cancer (CAC)-induced mice showed no significant difference between buparlisib treated and untreated group; B: In CAC-induced mice, buparlisib treatment demonstrated a significantly reduced expression of *Sgk2*. * $P < 0.05$ (compared to the control group); ° $P < 0.05$ (compared to the CAC untreated group); data are presented as mean $\pm$ SEM; one-way ANOVA was employed; $n = 4-8$. CAC: Colitis-associated cancer; DDCT: Delta-delta CT; *Pdk1*: Phosphoinositide-dependent kinase 1; *Sgk2*: Serum/glucocorticoid regulated kinase 2.

paradigm that has been widely used in CAC-related research[25,39]. A high DAI score indicates that the mouse CAC model is successful. The CAC-induced mice model manifested similar symptoms as CAC patients, where the mice were experiencing rectal bleeding, watering stool and body weight loss[40]. According to a previous study, symptoms including bloody stools, began to manifest between days 13 and 21 after the mice received DSS[41]. Similar results were shown in this study, where disease activity in CAC-induced mice began to increase dramatically following the second cycle of DSS, which occurred just 21 d after the first induction of DSS.

Histologically, three cycles of 2.5% DSS induced mild to moderate inflammatory response in the colitis group. No tumor formation was observed along the digestive system in the CRC group. Hence, no additional evaluation of those tissues was done. On the other hand, 50% of the CAC-induced mice group were found to have moderate inflammation. No diffuse inflammation was observed in the colonic mucosal of CAC-induced mice, even though it was reported that signs of diffuse inflammation can appear as early as third to fourth weeks after treatment[42]. Furthermore, compared to a prior study, where submucosal tumor infiltrations were sporadically seen in 20%–24% of AOM-DSS mice at week ten, the percentage of CAC-induced mice with tumors in this study was considered high[43]. The selection of the mouse strain may have affected the percentage of tumor occurrence because different strains of mice have varying susceptibilities.

Buparlisib was given daily for 14 d to the CAC-induced mice group to perform additional research on the function of PI3K inhibitors. Buparlisib is a potent oral pan-PI3K inhibitor that targets all four catalytic isoforms of class I PI3K (p110 α , p110 β , p110 δ and p110 γ)[44]. Buparlisib has been tested in clinical trials phase I and II in several solid tumors, including breast, brain, and lung cancer[20]. The preclinical studies showed buparlisib's inhibitory action against numerous PI3K isoforms and anti-proliferative effects in various *in vitro* cell lines[19]. Additionally, the authors provided the outcomes of *in vivo* studies with mouse xenograft models that demonstrate the effectiveness of buparlisib in reducing tumor growth [19]. Our findings showed that buparlisib treatment significantly decreased weight loss in CAC-induced mice. Another study supported this finding in which buparlisib therapy inhibited the growth of established patient-derived glioblastoma multiforme xenografts and increased survival in nude rats[45].

Additionally, buparlisib treatment in CAC-induced mice resulted in a slight rise in apoptotic cell activity and decreased cell proliferation. This result was consistent with several studies that found buparlisib to have the ability to induce apoptosis and reduce tumor growth in a variety of malignancies[44,46]. Buparlisib's effectiveness was also examined in CRC cell lines, where it was discovered that the drug had a considerable impact on the process of apoptosis induction, which progressed from AKT inhibition *via* FoxO3a-dependent p53 upregulated modulator of apoptosis induction[47].

Additionally, buparlisib treatment has been demonstrated to inhibit the PI3K/Akt signaling pathway, which causes apoptosis and slows tumor growth in medulloblastoma cells[44]. The most frequent downstream targets of buparlisib are AKT and mTOR. Studies have demonstrated that buparlisib effectively inhibited at serine 473 and threonine 308 and suppressed the phosphorylation of proteins downstream from mTORC1, including S6K and 4E-BP1 in bone and soft tissue sarcomas[48]. Despite that, there has been limited research conducted on the correlation between buparlisib and PI3K-non-AKT downstream pathways. Our results revealed that the non-AKT downstream of PI3K, *Pdk1* and *Sgk2* also showed a similar down-regulation tendency with buparlisib treatment. The expression of two PI3K-non-AKT downstream genes was previously found to be higher in malignant tissues than in healthy mucosa[49,50]. *PDK1* is a gene that plays a critical role in regulating cell proliferation and survival through its ability to curb signal propagation to the downstream targets such as AKT and *SGK2*. *PDK1* was discovered to interact with a specific protein known as HuR (human antigen R), which is known to attach to the mRNA of PI3K and stabilize it, enhancing the production of PI3K[51].

Meanwhile, *SGK* is activated by the same signaling cascade as *AKT*, and its inhibitor has been created and demonstrated encouraging results in preclinical tests as a possible therapeutic target in cancer[52]. The therapeutic potential of *SGK1* inhibition in cancer treatment has been demonstrated in preclinical studies using a variety of small molecule inhibitors and RNAi-based strategies that target *SGK1*[53]. Buparlisib and wortmannin, both of which are PI3K inhibitors, decreased the stimulatory impact of insulin on hOAT4 transport activity, aided by *SGK2*[54]. This showed that PI3K inhibition, whether by buparlisib or wortmannin, can lessen the stimulatory effect of insulin on hOAT4 transport activity and that *SGK2* may mediate this effect[54].

There are a few limitations in this study. There were only male mice used in the animal experiment. The AOM/DSS-induced mice model has been widely used in research, with a focus on male mice[42]. This preference for male mice is primarily caused by behavioural changes in female mice that can be altered by hormone cycles[55]. Additional studies using female balb/c mice will be required to establish that gender does not interfere with generalization of findings. Another limitation of this study is that the gene expression analysis was only performed on mouse tissues derived from inflammatory and malignant regions. It would be beneficial to include surrounding normal tissue in the analysis to observe and analyse any potential differences.

CONCLUSION

We investigated the functional relevance of the cytokine-induced PI3K-related gene variation, rs10889677, and it is hypothesized that this variant was a mediating factor in the beginning of the PI3K signaling pathway. Buparlisib, a PI3K inhibitor, was also administered, and it showed promise in preventing PI3K-non-AKT activation in the CAC-induced mice model. Overall, our research may offer a fresh perspective on how PI3K is used in the pathophysiology of CAC.

ARTICLE HIGHLIGHTS

Research background

The phosphatidylinositol-3-kinases (PI3K)/AKT pathway has emerged as a potential new approach in the complicated landscape of inflammation-related cancer, providing new hope for patients with colitis-associated cancer (CAC). A recent discovery in the investigation of PI3K-related genes revealed a promising association between ulcerative colitis (UC), colorectal cancer (CRC), and the elusive rs10889677 mutation.

Research motivation

Understanding the involvement of the PI3K signalling pathway in the development and progression of CAC. Genetic Variants and CAC Susceptibility: Investigating the impact of genetic variants, such as the rs10889677 variant, on CAC susceptibility and their contribution to PI3K pathway activation. The potential of the pan-class I PI3K inhibitor buparlisib as a potential therapeutic option for the treatment of CAC was being examined.

Research objectives

To investigate the role of PI3K-related gene variation in CAC pathogenesis and to evaluate the therapeutic potential of buparlisib, a powerful pan-class I PI3K inhibitor with tumor-suppressive properties.

Research methods

The study examined the genomic DNA from 32 colonic samples from cases of CAC, UC, and CRC. The rs10889677 mutation was highlighted, which was amplified and cloned for both mutant and wild-type fragments in the pmirGLO vector. Luciferase activity was measured using the HT29 cell line. CAC was induced using a precise protocol that included azoxymethane and sodium dextran sulphate. Buparlisib was administered after 14 d. Immunohistochemistry for Ki67 and Cleaved-caspase-3 markers as well as quantitative real-time polymerase chain reaction for *Pdk1* and *Sgk2* were performed on excised colonic tissues.

Research results

Buparlisib significantly reduced the mean weight loss in these mice, indicating an increase in general health and well-being. It has an effect in slowing cancer cell development and promoting cell death in CAC-induced mice. It also had an effect on the expression of particular genes involved in the PI3K pathway, with significantly decreased *Sgk2* expression and a decreasing trend in *Pdk1* expression.

Research conclusions

Our study discovered that a specific genetic variant (rs10889677 variation) is critical in the activation of a cancer-promoting pathway known as PI3K. We also discovered that a drug called buparlisib can prevent this route from being triggered, which is great news for those fighting cancer (CAC).

Research perspectives

Patient stratification: Future research could look into whether the rs10889677 variant can be used as a biomarker to

predict CAC development in UC patients. This could help with early detection and personalised treatment initiatives. Mechanism Elucidation: The mechanisms by which the rs10889677 variation alters the PI3K pathway should be studied further in the future.

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FOOTNOTES

Author contributions: Mokhtar NM and Raja Ali RA contributed to the conceptualization; Mokhtar NM, Raja Ali RA, Muhammad Nawawi KN, and Razali NN contributed to the methodology and participant recruitments; Razali NN, and Mohd Rathi ND contributed to the animal handling; Razali NN, Yahaya A, and Mokhtar NM performed the validation; Razali NN, and Mokhtar NM contributed to the data analysis; Razali NN wrote the original draft preparation; Mokhtar NM, Raja Ali RA, and Muhammad Nawawi KN wrote the review and editing; Mokhtar NM, Raja Ali RA, and Muhammad Nawawi KN contributed to the supervision; All authors have read and agreed to the published version of the manuscript.

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Country/Territory of origin: Malaysia

ORCID number: Nurul Nadirah Razali 0000-0002-7725-0353; Raja Affendi Raja Ali 0000-0003-4995-3868; Khairul Najmi Muhammad Nawawi 0000-0002-5309-5301; Norfilza Mohd Mokhtar 0000-0002-4863-100X.

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