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Molecular chemoprevention by morin – A plant flavonoid that targets nuclear factor kappa B in experimental colon cancer

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ABSTRACT

Colorectal cancer is the third most common cancer worldwide. The development of effective, inexpensive and safe chemopreventive agents would be of great benefit as it involves use of natural products to prevent or suppress the progression of precursor lesions. Morin a flavonoid found in figs (*Ficus carica*) and other plants is shown to inhibit 1,2-dimethylhydrazine (DMH) induced colon cancer progression in a short term and long term model of colon cancer rats; however, the molecular target for the colon cancer chemoprotective efficacy of morin is yet to be discovered. The present study aims to explore the molecular basis of how morin contributes to the chemoprevention with a focus on NF-κB signaling pathway. The effect of morin on NF-κB signaling in DMH-induced carcinogenic events such as inflammation and apoptosis were analyzed by studying the histopathological analysis using Hematoxylin and Eosin staining (H &E), mRNA expression using q-PCR, protein expression using Immunohistochemistry (IHC) and western blot. Morin supplementation to DMH administered rats down regulated NF-κB pathway and its downstream inflammatory mediators like tumor necrosis factor alpha (TNF-α), interleukin 6 (IL-6), cyclooxygenase 2 (COX-2) and prostaglandin (PGE-2). Morin supplementation to DMH administered rats alters BAX/BCL2 ratio favoring apoptosis in carcinogen treated rats. Our findings explored that molecular chemoprevention of morin targets NF-κB and acts as a potent anti-inflammatory and pro-apoptotic agent for colon cancer prevention.

1. Introduction

Nuclear factor of κB (NF-κB) is a protein complex generated through combinations of five protein members (RelA/p65, RelB, c-Rel, p50/p105, and p52/p100) that bind a common sequence motif known as the κB site [1]. Of all the NF-κB family members Rel A (p65), Rel B, c-Rel are synthesized as matured proteins whose N terminal end has Rel homology domain (RHD) required for dimerization, DNA binding and C terminal end harboring transcription modulating domains which is lost during their nascent proteolytic processing of p105, and p100 to become functional p50 and p52 respectively [2,3]. The activity of these homo or heterodimers is regulated by two main pathways [4]. The first canonical pathway includes activation of RelA (p65), C-Rel and p50 in response to microbial or viral infections that lead to activation of IκB kinase. The second non-canonical pathway is induced by the members of the tumor necrosis factor (TNF) cytokines that activates IKKα along with NF-κB inhibitor kinase (NIk) which in turn leads to phosphorylation mediated proteolytic processing of NFκB2 that results in dimerization and nuclear translocation of RelB-p52. Both the pathways regulate the expression of genes involved in immunoregulation, inflammation, cell proliferation, apoptosis and genes that regulate negative regulators of NF-κB [5]. Constitutive NF-κB activation and addiction of cancer cells to activated NF-κB has been implicated in every stage of tumorigenesis and it involves a wide range of mechanisms including fusion proteins, IκB mutations, IKK hyperactivity, overexpression of ligands and receptors and mutations in NF-κB [6,7]. Colorectal carcinogenesis (CRC) is a multistep process characterized by genetic/epigenetic alterations in oncogenic Ras and p53 [8]. About 66% of colon cancer cell lines and 40% of human CRCs showed constitutive NF-κB activation [9,10]. Experimental data pinpoint the involvement of NF-κB in colorectal carcinogenesis [11]. Tumor suppressor p53, one of the most important genes mutated in CRC is

Abbreviations: NF-κB, Nuclear factor kappa B; DMH, dimethylhydrazine; TNF-α, tumor necrosis factor alpha; IL-6, interleukin 6; COX-2, cyclooxygenase 2; PGE-2, prostaglandin

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responsible for the suppression of the inflammatory milieu generated by NF-κB in a mouse model of CRC [12].

NF-κB contributes to colon carcinogenesis by deregulating the homeostasis between intestinal epithelial cells (IECs) and gut micro biota that results from raised plasma levels of lipopolysaccharide due to abnormal function of the colonic epithelial barrier after the influence of carcinogens [13]. Increased NF-κB activity is also known to obstruct radiation induced cancer cell death and develops chemo resistance to anticancer treatment. NF-κB activation can be inhibited by many ways including the modulation of protein kinases, protein phosphatases, proteasome inhibitors, blocking nuclear translocation, p65 acetylation, blocking DNA binding etc. But due to its ubiquitous nature, NF-κB targeting drugs show serious side effects, which limit their clinical use.

Natural products and their derivatives are always preferred for the treatment and prevention of chronic diseases including cancer due to their less or no adverse effects. Morin, a bioactive flavonoid isolated from the family members of Moraceae has been reported to possess several pharmacological properties [14]. Morin is shown to protect gastric mucosa from indomethacin induced tissue damage by modulating NF-κB [15]. Morin supplementation for a period of 15 weeks significantly reduced the colonic preneoplastic lesions in rats challenged with the colon specific pro carcinogen DMH [16]. Recent research from our laboratory explored that morin exhibited more potential chemopreventive efficacy than esculetin in a long term model of colon carcinogenesis[17]. The present study aims to explore the molecular basis of how morin contribute to the chemoprevention with a focus on NF-κB signaling pathway.

2. Materials and methods

2.1. Chemicals

1,2-Dimethyl hydrazine (DMH), morin, RNA later and polymerase chain reaction (q- PCR) primers (Table 1) were purchased from Sigma Aldrich (St.Louis, MO, USA). RNAiso plus was purchased from TaKaRa (Japan). cDNA synthesis kit and SYBR green were purchased from Bio line (Bio line, USA). RIPA lysis buffer and protease inhibitor cocktail were purchased from (Thermo Fisher Scientific, U.S.A). The pre-diluted antibodies for BAX, BCL2 and polymer HRP detection kit were purchased from BioGenex laboratories (San Ramon, CA, USA). The NF-κB pathway sampler kit (#9936) comprising antibodies for NF-κB, p-NF-κB, IKKα, IKKβ, p-IKKα/β, IκBα, p-IκBα and β-actin were purchased from cell signalling (U.S.A). ECL detection kit was purchased from (Bio-Rad, USA). All other chemicals and solvents were purchased from Hi-Media Laboratories (Mumbai, Maharashtra, India).

Table 1
List of primers.

Gene	NCBI accession Number	Primer
IL-6	NM_012589.2	Forward primer: CCGGAGAGGAGACTTCACAG Reverse primer : ACAGTGCATCATCGCTGTTC
TNF-α	NM_012675.3	Forward primer: AGTCCGGGCAGGTCTACTTT Reverse primer : GGCCACTACTTCAGCGTCTC
COX-2	S67722.1	Forward primer: ACTTGCGTTGATGGTGGCTGTCTT Reverse primer :CTGTATCCCGCCCTGCTGGTG
PGE-2	NM_031088.1	Forward primer: CTTTAGTCTGGCCACGATGC Reverse primer :ACAGAAGAGCAAGGAGACCC
β-actin	NM_031144.3	Forward primer: GAGAGGGAAATCGTGCGTGAC Reverse primer: CATCTGCTGGAAGGTGGACA

2.2. Animals and ethical clearance

Male albino Wistar rats (130–150 g) were procured from the Central Animal Facility, SASTRA Deemed University, Thirumalaisamudram, Thanjavur, Tamil Nadu, India. All the rats were accommodated in polypropylene cages with the paddy husk for bedding. The room temperature was maintained at 22 ± 3 °C and relative humidity of 30–70% with 12-h light/ 12-h dark cycle. Rats were fed with standard diet pellet (Altromin) and RO water ad libitum. All the experimental studies involving the use of animal or animal tissue were performed in accordance with the Indian National Law on animal care and use approved by the Institutional Animal Ethics Committee of SASTRA University (CPCSEA approval no:276/SASTRA/IAEC/RPP).

2.3. Phytochemical and inducer administration

Morin at a dose of 50 mg/kg [17] was weighed and suspended in 0.2% (w/v) of carboxymethylcellulose (CMC) and was given every day via intragastric intubation for a period of 30 weeks. 1, 2- dimethylhydrazine at a dose of 40 mg/kg b.w. was calculated [18], weighed and dissolved in 1 mM ethylene diamine tetra acetic acid (EDTA) to make sure the firmness of the chemical just prior to use and the pH was adjusted to 6.5 with 1 mM Sodium Hydroxide (NaOH). Subcutaneous injections of DMH were given to rats once a week for 10 weeks starting from the 4th week of the experimental period of 30 weeks.

2.4. Experimental design

The rats were randomized into four experimental groups (n = 6/group). Group 1 rats were given 0.2% CMC and served as normal control. Group 2 rats were given morin and served as morin control. Group 3 rats were given DMH alone and served as DMH control. Group 4 rats were given morin and DMH. The body weights of the rats were measured every week throughout the experimental period. At the end of the experimental period the rats were sacrificed by CO_2 asphyxiation and subjected to necropsy.

2.5. Sample collection and processing

For histological studies excised colon was collected and post fixed in 4% para formaldehyde for 48 h. The tissues were processed using automated tissue processor and embedded in paraffin using embedded station with cold plate (Leica TP 1020, Leica EG1150, Leica Biosystems, Wetzlar, Germany) and cut into 3 µm sections using microtome (Leica RM 2125 RTS, Leica Biosystems, Wetzlar, Germany). For q-PCR experiment the tissues were finely minced and stored at −20 °C in RNA later for the q-PCR study. For western blotting, the tissues were snap frozen in liquid nitrogen and stored at −80 °C till further use.

2.6. Histological studies

Hematoxylin and Eosin staining was done using an automated tissue processing and staining system (Leica TP 1020; Leica EG1150; Leica RM 2125 RTS and Leica ST4040). The tissue sections were mounted in DPX and visualized under PC connected binocular microscope with inbuilt camera (Nikon Eclipse Ci-Ds-Fi2).

2.7. Immunohistochemistry studies

The 3 µm thin paraffin-embedded sections were heated for 1 h at 60 °C, deparaffinised in xylene and rehydrated with graded alcohols (100%, 70%, and 50%) at room temperature. Tissue section slides were heated in pressure cooker containing trisodium citrate buffer solution (pH 6.0). The slides were allowed to cool and washed with tris buffer saline (pH 7.6) followed by adding the power block and peroxidase block according to the manufacturer's instructions (Biogenex, San