Calcium Prevents Tumorigenesis in a Mouse Model of Colorectal Cancer

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Abstract

Background and Aim: Calcium has been proposed as a mediator of the chemoprevention of colorectal cancer (CRC), but the comprehensive mechanism underlying this preventive effect is not yet clear. Hence, we conducted this study to evaluate the possible roles and mechanisms of calcium-mediated prevention of CRC induced by 1,2-dimethylhydrazine (DMH) in mice.

Methods: For gene expression analysis, 6 non-tumor colorectal tissues of mice from the DMH + Calcium group and 3 samples each from the DMH and control groups were hybridized on a 4×44 K Agilent whole genome oligo microarray, and selected genes were validated by real-time polymerase chain reaction (PCR). Functional analysis of the microarray data was performed using KEGG and Gene Ontology (GO) analyses. Hub genes were identified using Pathway Studio software.

Results: The tumor incidence rates in the DMH and DMH + Calcium groups were 90% and 40%, respectively. Microarray gene expression analysis showed that 5100 genes were differentially expressed compared with the DMH + Calcium group. Functional analysis showed that the Wnt, cell cycle, and arachidonic acid pathways were significantly downregulated in the DMH + Calcium group, and that the GO terms related to cell differentiation, cell cycle, proliferation, cell death, adhesion, and cell migration were significantly affected. Forkhead box M1 (FoxM1) and nuclear factor kappa-B (NF-κB) were considered as potent hub genes.

Conclusion: In the DMH-induced CRC mouse model, comprehensive mechanisms were involved with complex gene expression alterations encompassing many altered pathways and GO terms. However, how calcium regulates these events remains to be studied.

Introduction

The current incidence of colorectal cancer (CRC) is high, and its prognosis remains poor [1]; hence, it is important to devise strategies to prevent the development of CRC. Many agents are used to prevent CRC [2]; however, there is no consistency in the effects across agents [3,4,5], and some agents may lead to severe side effects [6,7]. Thus, an alternative approach is necessary for the prevention of CRC.

A recent systemic review revealed that calcium may be more attractive in the prevention of CRC compared with aspirin and screening [8]. However, the effectiveness of calcium is still uncertain. Pooled findings of 10 cohort studies revealed a reverse relationship between calcium intake and CRC risk [9], but this relationship was not confirmed by a randomized controlled clinical trial [10]. However, due to the limitations of the randomized control trial (RCT), e.g., low dose of calcium, short length of follow-up, and influence of estrogen intake [11], the conclusion from the RCT was not convincing. Nonetheless, the effectiveness of calcium on the prevention of CRC remains controversial [12].

Similarly, the mechanism of calcium-mediated prevention of CRC is not yet clear. In previous studies, calcium was thought to reduce the risk of CRC by binding to toxic secondary bile acids and forming insoluble soaps in the lumen of the colon [13], or by reducing proliferation, stimulating differentiation, and inducing apoptosis in the colonic mucosa [14,15]. A recent study suggested that calcium could abrogate hyperplasia in the distal colon by inhibiting a calcium channel receptor, i.e., transient receptor potential channel, subfamily V, member 6 (TRPV6) [16]. These findings indicate that calcium could reduce the risk of CRC through a variety of mechanisms, but the specific and comprehensive nature of its action remains to be elucidated.

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mechanism is yet not clear. Therefore, we conducted this study to evaluate the possible roles and mechanisms of calcium-mediated prevention of CRC induced by 1,2-dimethylhydrazine (DMH) in mice. We believe that our findings will contribute to the research on the clinical application of calcium.

Materials and Methods

Ethics Statement

Our study was approved by the Animal Care and Use Committee of the Shanghai Jiao-Tong University School of Medicine Renji Hospital, Shanghai, China. All animal procedures were performed according to the guidelines developed by the China Council on Animal Care and the protocol approved by the Shanghai Jiao-Tong University School of Medicine, Renji Hospital, Shanghai, China.

Chemicals

DMH was obtained from Sigma Chemical Co. (St. Louis, MO, USA). Normal and high calcium feed for mice were obtained from Shanghai SLAC Laboratory Animal Company (Shanghai, China). The main components of the normal and high calcium feed were the same: protein, 22.1%; fat, 5.28%; ash, 3.2%; fiber, 4.12%; nitrogen-free extract, 52%; phosphorus, 0.92%; and Vitamin D3 6818.4 IU/kg. The concentration of calcium (in the form of calcium carbonate) in the normal and high calcium feed was 1.24% and 3.0%, respectively. The high calcium feed was adjusted by adding calcium carbonate to obtain a final calcium content of 3%. Because we think that the controversial effectiveness of high calcium diet on the prevention of CRC may be partially due to the small dose of calcium in previous studies, so we decided to use higher calcium content to test the exact preventive effect of CRC and the mice’s tolerance.

Experimental animals

In total, 80 female Slac: ICR mice [weight, 18–20 g; grade, specific pathogen-free (SPF)] were purchased from the Chinese Academy of Sciences (Shanghai, China). They were maintained at room temperature (22°C) with a relative humidity of 60% and 12-hour light/dark cycles; they were supplied a standard laboratory diet and drinking water. The mice were randomly divided into 4 groups (20 mice in each group): Control group, DMH group, DMH+Calcium group, and Calcium group. The mice of the Control group were provided normal feed and received subcutaneous injections of normal saline; those of the DMH group were provided normal feed and received subcutaneous injection of DMH at a dose of 20 mg/kg once weekly for 20 weeks; those of the DMH+Calcium group were provided high-calcium feed and received subcutaneous injection of DMH at a dose of 20 mg/kg once weekly for 20 weeks; those of the Calcium group were provided high-calcium feed and received subcutaneous injections of normal saline. The mice were killed at the end of 24 weeks, and the incidence of CRC in each group was examined. This method has been described in detail in our previous study [17]. In brief, longitudinal incisions were made in colorectal tissues to observe the number of gross tumors. After the tumors were removed separately and the full-thickness colorectal tissues were cutted in half by the longitudinal axis. Some of the freshly obtained tumor samples together with their half corresponding non-tumor colorectal tissues were frozen immediately in liquid nitrogen. The remaining samples were fixed in formalin solution and embedded in paraffin blocks for pathological analysis and immunohistochemistry.

Histological analysis

For histological analysis, 4-μm-thick sections of formalin-fixed, paraffin-embedded colon tumors were prepared. After hematoxylin and eosin staining, the sections of each tumor were examined under a light microscope (Olympus, Japan). The results were confirmed by the pathologist Chen XY.

RNA extraction, labeling, hybridization, and analysis

Total RNAs from 12 non-tumor colorectal tissues [3 from the Control group, 3 from the DMH group, 6 from the DMH + Calcium group (among the 6 from the DMH + Calcium group, 3 were from mice with tumors and 3 were from mice without tumors)] were harvested using TRIzol (Invitrogen) according to manufacturer’s instruction. The RNA content was measured using a Nanodrop ND-1000 spectrophotometer, and denaturing gel electrophoresis was conducted. Next, the samples were amplified, labeled using the Agilent Quick Amp labeling kit, and hybridized using the Agilent whole genome oligo microarray (Agilent Technologies, Palo Alto, CA) by using Agilent SureHyb hybridization chambers. After hybridization and washing, the processed slides were scanned with the Agilent DNA microarray scanner using the settings recommended by Agilent Technologies.

The resulting text files extracted from the Agilent Feature Extraction Software (version 10.5.1.1) were imported into the Agilent GeneSpring GX software (version 11.0) for further analysis. Background intensity has been cut off before normalization. The microarray data sets were normalized in GeneSpring GX using the Agilent FE one-color scenario (mainly median normalization). Differentially expressed genes were identified by determining the fold-change (FC) and p values of the t-test. Genes with an FC of ≥1.5 and a p value of ≤0.05 between 2 groups were identified as differentially expressed genes. Functional analysis of the differentially expressed genes was performed using Gene Ontology (GO) (http://www.geneontology.org/) [18] and the KEGG PATHWAY Database (http://www.genome.jp/kegg/pathway.html). Hub genes were identified using Pathway Studio (Ariadne Genomics) [19].

Real-time polymerase chain reaction

Reverse transcription was performed using oligo (dT) primers and superscript II reverse transcriptase (Takara). Quantification of gene expression was performed using a real-time polymerase chain reaction (PCR) kit (Takara). The expression level of 18 s rRNA was used as an internal control. The expression of the following genes was analyzed: S100a9, Defa20, Mmp10, Prx2, Pgr, Rnf152, and Prdx6. The primers are listed in Table 1.

Immunohistochemistry

Four-micrometer-thick sections of formalin-fixed, paraffin-embedded non-tumor colon tissues were deparaffinized and rehydrated. The microwave repair method was used for antigen retrieval. Endogenous peroxidase activity was blocked by incubation of the sections with 0.3% hydrogen peroxide for 10 min. Non-specific antigen was blocked by incubation of the sections with sheep serum for 30 min. Slides were incubated overnight at 4°C in a humidified chamber with rabbit monoclonal anti-β-catenin (Cell Signaling Technology, #9562) at a dilution of 1:200. Anti-rabbit IgG was used as secondary antibody (30 min incubation). Diaminobenzidine was used as chromogen and the sections were counterstained with hematoxylin. Samples incubated with PBS instead of primary antibody were used as negative controls.
Table 1. Primer sequence.

| Gene name | Forward sequence | Reverse sequence | Product length |
|-----------|------------------|------------------|----------------|
| S100a9    | 5’-AACATCTGGACTTATTACGCTTG-3’ | 5’-ACTGTGCTTCCACATTGTCCT-3’ | 169            |
| Mmp10     | 5’-CCAGGTCACTCTCCACCTTTT-3’ | 5’-AGGACAGATCACTTGTCCATG-3’ | 142            |
| Defa20    | 5’-AGGTGGGATCTGAACTATGTA-3’ | 5’-GATGGAATCTGCTGAGTAAAG-3’ | 153            |
| Ptg2      | 5’-GAGTTGGGATCTGAACTATGTA-3’ | 5’-GATGGAATCTGCTGAGTAAAG-3’ | 153            |
| Per3      | 5’-CACTCTCTCAGGATTGCCACACTT-3’ | 5’-CCAGATCCGCTTTCATTGAGTG-3’ | 126            |
| Tef       | 5’-TTTCTCTCTGCTGACATACCTTTCA-3’ | 5’-GGTGTACGCTCTTTAGAGTG-3’ | 106            |
| Rnf152    | 5’-AGTCAATGCTACACAGCACTC-3’ | 5’-GACAGAGATGATCCATTTGCC-3’ | 120            |
| Prdx6     | 5’-AGTGGACAGACAGACAGCAAC-3’ | 5’-GACAGAGATGATCCATTTGCC-3’ | 150            |

Statistical analysis
The results of the animal experiments and real-time PCR were analyzed using SAS 9.2 software (SAS Institute Inc. USA). Data are presented as means ± SD. Student’s t-test was used to compare values between 2 independent groups.

Results
Animal experiments
The main results of the animal experiments are shown in Figure 1. We successfully induced CRC in mice using DMH (Figure 1A). Most of the cancers were identified as adenocarcinomas by histological analysis (Figure 1B, 1C). In the DMH and DMH+Calcium groups, the tumor incidence was 90% and 40% (Figure 1D), the mean number of tumors per mouse was 5.39 ± 1.97 and 3.0 ± 1.31 (Figure 1E), and mean maximum diameter of the tumors was 6.44 ± 1.72 and 2.63 ± 1.19 (Figure 1F), respectively. No tumor was found in the Control and Calcium groups. Growth and development of the mice in the Calcium group were not significantly affected, and the pathological examination of their kidneys, hearts, lungs, livers, and spleens revealed no obvious abnormalities (data not shown).

Gene expression profile by microarray analysis
All 12 colonic tissues cleared the quality control step and were analyzed as described in the Methods section. The microarray analysis was conducted between the Control group (3 samples), DMH group (3 samples), and DMH + Calcium group (6 samples). We also compared the gene expression levels between the samples with or without tumors among the DMH + Calcium group.

Hierarchical clustering analysis of the 12 array expression data showed a homogenous expression profile among the samples of each group (Figure S1). By setting the filter for the FC to ±1.5 and the p value at ≤0.05, we found that the expression of 12395 genes was significantly altered in the DMH group compared to those in the Control group (see Table S1), and that of 1508 genes was significantly altered in the DMH + Calcium group compared to those in the DMH group (see Table S2). Compared with Table S1 and Table S2, we found that the expression of 549 genes whose changes due to DMH treatment could be reversed by dietary calcium (see Table S3). In Table 2, the 30 most differentially expressed genes are shown, which are clearly related to tumorigenesis, such as S100a9 (S100 calcium-binding protein A9 [calgranulin B]), Defa20 (defensin alpha 20), Mmp10 (matrix metalloproteinase 10), Ang2 (angiogenin, ribonuclease A family, member 2), Per3 (period homolog 3), Tef (thyrotrph embryonic factor), and Ptg2 (prostaglandin-endoperoxide synthase 2). For the 8 selected genes, i.e., S100a9, Defa20, Mmp10, Ptg2, Per3, Tef, Rnf152, and Prdx6, the results obtained from the microarray analysis were confirmed by real-time PCR (Figure 2). We selected these genes for PCR confirmation because they are closely related to tumorigenesis and worth further research.

To determine whether specific biological pathways or functional gene groups were differentially affected by the high calcium diet, we analyzed our microarray dataset (on the basis of the results shown in Table S3) using the GO and KEGG software. The detailed results are shown in Table S4 and Table S5. By setting the p value at ≤0.05, we found significant downregulation of 39 signaling pathways, including some tumor-related pathways such as the cell cycle pathway, Wnt signaling pathway, vascular endothelial growth factor (VEGF) signaling pathway, and transforming growth factor (TGF-β) signaling pathway. The most enriched pathways are shown in Table 3. We used immunohistochemistry to detect the protein expression and distribution of β-catenin, the core molecule in the Wnt pathway, and found that its expression was significantly higher in the non-tumor colonic mucosa from the DMH group than in that from the Control group, however, its expression in the DMH + Calcium group was markedly reduced compared with the DMH group (Figure 3).

Altogether, 703 GO terms, including tumor-related GO terms such as cell differentiation, cell proliferation, apoptosis, angiogenesis, cell adhesion, cell cycle, and cell division, were significantly changed.

We also used Pathway Studio software to filter hub genes that may play key roles in the calcium-mediated prevention of CRC (Table 4). Of those, 2 genes were selected to construct gene networks (Figure 4). On the basis of the networks, we could clearly determine the core roles of the hub genes.

In addition, we further compared the gene expression levels among the mice from the DMH + Calcium group with/without tumors and found that the gene expression levels of most of the above selected genes in mice from the DMH + Calcium group with tumors was between those from the DMH + Calcium group without tumors and those from the DMH group (see Table S6 and Figure S1). We also found that the expression of some calcium transporters was different among the mice from the DMH + Calcium group with/without tumors. For example, the expression of Trpv6 and Trpv3 was significantly upregulated in mice from the DMH + Calcium group with tumors compared to those without tumors; however, the expression of plasmalemna Ca2+-ATPase (PMCA) was significantly downregulated.
Discussion

In this study, we used the DMH-induced CRC mouse model that shows phenotypic and genotypic features similar to those observed in human sporadic CRCs and, thus, can be applied to study the prevention of CRC [20]. In this study, we found that the incidence of CRC decreased significantly in mice fed a high calcium diet, indicating a clear role of calcium in the prevention of CRC. Our results are consistent with many other studies. Pence [21] have found that rats on the high calcium diet have a decreased CRC incidence compared to the low calcium diet (86% vs. 53%), Salas [22] also observed a significant diminution in the number of tumors and a decrease in the CRC incidence in the group fed high calcium diet (97% vs. 64%). However, some studies have observed different outcomes. Sitrin [23] found that neither calcium supplementation alone nor supplemental calcium con- junction with vitamin D deficiency altered the incidence of CRC induced by DMH, Mølck [24] even found an increased incidence in the rats fed high calcium diet. We think that the differences in the animal strain, animal age, rearing environment, basis diet, DMH doses and duration of injection, calcium concentration, calcium intervention time may be accounted for the differences in the outcome of various studies using high calcium. The confirmed preventive effectiveness of CRC with high calcium diet could not yet be contained from those studies. We think it is may be due to the small doses of calcium in those studies. So we used higher calcium content(3%) in the feed to test the preventive effect of CRC and the mice' tolerance. In fact, this high calcium feed showed good preventive effect of CRC in our experiment and no obvious adverse effects was found in mice. In addition, no tumor was found in the Calcium groups. Growth and development of the mice in the Calcium group were not significantly affected, and the pathological examination of their kidneys, hearts, lungs, livers, and spleens revealed no obvious abnormalities, the expression of most of the selected genes confirmed by real-time PCR in the Calcium group was equal to that in the Control group. These findings indicate that this high calcium diet is safe for the mice.

Next, we used microarray gene expression profile analysis to determine the mechanism of calcium-mediated prevention of CRC. To the best of our knowledge, this is the first investigation to use microarray technology for studying the role of high calcium diet in the prevention of CRC.

When the FC was set to ≥1.5, the changes in 549 genes that were the result of DMH treatment could be reversed with dietary calcium. S100a9 is the most downregulated gene; its product is a cytoplasmic Ca$^{2+}$-binding protein. Although extracellular S100a9 could induce apoptosis by binding to a yet unknown receptor and cause dimerization of Bax and Bak and their translocation to the mitochondria leading to mitochondrial damage [25], its expression in epithelial cells could indeed induce cell proliferation by activating the phosphoinositide 3-kinase (PI3K)–Akt–NF-κB survival pathway, which is associated with tumorigenesis [26].
addition, S100a9 is an important proinflammatory cytokine involved in innate immunity, and its expression was found to be elevated in numerous pathological conditions associated with inflammation [27]. Many other inflammatory cytokines, such as Defa, were also significantly downregulated [members of the defensin family were downregulated at different levels: Defa-rs2 (FC = -9.88), Defa20 (FC = -8.16)]. Tumor necrosis factor (Tnf) and its receptors were also downregulated at different levels: Tnf (FC = -1.87), Tnfsf11b (FC = -5.98), Tnfsf19 (FC = -2.27). Several other studies performed using the DMH or azoxymethane (AOM)-induced CRC model also found high expression levels of S100a9 and Defa in tumor tissues [28,29]. Inflammatory cytokines play important roles in the innate and adaptive immunity; however, those elevated cytokines are also involved in tumor initiation, promotion, and invasion [30,31,32,33], and thus, play an important role in inflammation-associated carcinogenesis [34].

MMPs are a family of matrix metalloproteinases that can play important roles in the tumor microenvironment [35]. Many members of the MMP family were significantly downregulated in the DMH + Calcium group [MMP10 (FC = -9.47), MMP13 (FC = -9.1), MMP7 (FC = -7.07), and MMP11 (FC = -5.6)]. Other metalloproteinases were also downregulated, such as Adam8 and Adam10, which were downregulated by 2.08 and 1.6 times, respectively. Recent studies have shown that many members of the MMP and ADAM family could play various roles in tumorigenesis. For example, MMP7 could cleave Fas ligand, thereby lowering the impact of chemotherapy on the tumor by abrogating apoptosis [36]. Adam10 may trigger the release of soluble epidermal growth factor (EGF), mediate the shedding of E-cadherin, translocating β-catenin to the nucleus, and thus, driving cell proliferation [37]. However, the most important role of the members of the MMP and ADAM family is promoting tumor invasion and metastases by degrading extracellular matrix [38,39]. These findings indicate that a high calcium diet may play an important role in inhibiting invasion and metastases of CRC. Because in vitro studies showed that, in CRC cells, E-cadherin

| Accession number | Gene symbol | Gene Description | Fold change | P value |
|------------------|-------------|-----------------|-------------|---------|
| NM_007847        | Defa-rs2    | defensin, alpha, related sequence 2 | -9.88       | 1.93E-04|
| NM_009114        | S100a9      | S100 calcium binding protein A9 | -9.58       | 4.25E-05|
| NM_019471        | Mmp10       | matrix metallopeptidase 10 | -9.47       | 7.67E-07|
| NM_008607        | Mmp13       | matrix metallopeptidase 13 | -9.10       | 1.48E-04|
| NM_183268        | Defa20      | defensin, alpha, 20 | -8.16       | 1.20E-04|
| NM_010810        | Mmp7        | matrix metallopeptidase 7 | -7.07       | 0.0041107|
| EU817850         | Gm5222      | monoclonal antibody 1D6 immunoglobulin light chain variable region | -6.98       | 1.99E-05|
| NM_008764        | Tnfsf11b    | tumor necrosis factor receptor superfamily, member 11b | -5.98       | 0.01292794|
| NM_011915        | Wif1        | Wnt inhibitory factor 1 | -5.79       | 6.00447852|
| NM_00174099      | Krt36       | keratin 36 | -5.79       | 2.75E-04|
| NM_009263        | Spp1        | secreted phosphoprotein 1 | -5.40       | 1.31E-04|
| NM_175391        | Apol7c      | apolipoprotein L 7c | -4.93       | 5.03E-07|
| NM_007969        | Expi        | extracellular proteinase inhibitor | -4.78       | 3.00E-02|
| NM_008256        | Hmgcr2      | 3-hydroxy-3-methylglutaryl-Coenzyme A synthase 2 | -4.62       | 9.86E-06|
| NM_010743        | Iilr1f      | interleukin 1 receptor-like 1 | -4.38       | 1.28E-05|
| NM_026414        | Asprv1      | aspartic peptidase, retroviral-like 1 | -4.38       | 2.82E-05|
| NM_007449        | Ang2        | angiogenin, ribonuclease A family, member 2 | -4.34       | 0.04452419|
| NM_053080        | Adh1h3a     | aldehyde dehydrogenase family 1, subfamily A3 | -4.11       | 0.01939012|
| NM_007440        | Alox12      | arachidonate 12-lipoxygenase | -4.03       | 0.02319294|
| NM_00142959      | Bcl2l15     | BCL2-like 15, transcript variant 1 | -3.95       | 5.27E-04|
| NM_016958        | Krt14       | keratin 14 | -3.81       | 0.00713059|
| NM_007753        | Cpa3        | carboxypeptidase A3 | -3.74       | 3.03E-05|
| NM_008939        | Prss12      | protease, serine, 12 neurotyspin | -3.65       | 0.04354441|
| NM_011280        | Trim10      | tripartite motif-containing 10 | -3.58       | 3.46E-06|
| NM_001082331     | Pla2g2a     | phospholipase A2, group IIA | -3.50       | 8.61E-04|
| NM_021285        | Myl1        | myosin, light polypeptide 1 | -3.31       | 9.72E-04|
| NM_011067        | Per3        | period homolog 3 | 3.16        | 2.08E-05|
| NM_0153484       | Tef         | thyrotroph embryonic factor | 2.98        | 1.26E-05|
| NM_011198        | Ptgs2       | prostaglandin-endoperoxide synthase 2 | -2.87       | 1.53E-04|
| AK081980         | Prdx6       | 16 days embryo head cDNA, product: peroxiredoxin 5 | 2.87       | 0.01674954|
| NM_178779        | Rnf152      | ring finger protein 152 | 2.63       | 0.00288787|

Table 2. List of the most differentially expressed genes whose changes due to DMH treatment could be reversed by dietary calcium.

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Figure 2. Validation of differentially expressed genes by real-time polymerase chain reaction. We used 18 s rRNA as an internal control. Relative mRNA expression was calculated according to the $2^{-\Delta\Delta C_{T}}$ method. Data are the mean of 10 samples ± SD. Control, gene expression level in the normal colon tissue of mice in the Control group; DMH, gene expression level in the non-tumor colon tissue of mice in the DMH group; DMH+Calcium, gene expression level in the non-tumor colon tissue of mice in the DMH+Calcium group; Calcium, gene expression in the non-tumor colon tissue of mice in the Calcium group. * $P$ value<0.05 between DMH+Calcium group and DMH group; § $P$ value<0.05 between Calcium group and Control group.

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Table 3. The most enrichment pathways by KEGG.

| Pathway ID | Pathway name                                | Selection Count | Count  | Enrichment  |
|------------|---------------------------------------------|-----------------|--------|-------------|
| **Down-regulated pathways** | | | | |
| mmu04310  | Wnt signaling pathway                       | 11              | 148    | 5.123285    |
| mmu05140  | Leishmaniasis                               | 6               | 67     | 3.468583    |
| mmu04110  | Cell cycle                                  | 8               | 127    | 3.383628    |
| mmu04370  | VEGF signaling pathway                      | 5               | 76     | 2.378304    |
| mmu04010  | MAPK signaling pathway                      | 10              | 272    | 2.299372    |
| mmu04114  | Oocyte meiosis                              | 6               | 114    | 2.277315    |
| mmu04664  | Fc epsilon RI signaling pathway             | 5               | 81     | 2.260629    |
| mmu04350  | TGF-beta signaling pathway                  | 5               | 84     | 2.194259    |
| mmu05222  | Small cell lung cancer                      | 5               | 84     | 2.194259    |
| mmu04640  | Hematopoietic cell lineage                  | 5               | 85     | 2.172789    |
| mmu00590  | Arachidonic acid metabolism                 | 5               | 86     | 2.151633    |
| mmu04012  | ErbB signaling pathway                      | 5               | 88     | 2.110233    |
| mmu00650  | Butanoate metabolism                        | 3               | 30     | 2.084872    |
| mmu05216  | Thyroid cancer                              | 3               | 30     | 2.084872    |
| **Up-regulated pathways** | | | | |
| mmu00982  | Drug metabolism - cytochrome P450            | 8               | 85     | 7.972006    |
| mmu04710  | Circadian rhythm - mammal                   | 4               | 22     | 5.391566    |
| mmu00591  | Linoleic acid metabolism                    | 3               | 45     | 2.827674    |
| mmu00910  | Nitrogen metabolism                         | 2               | 23     | 2.231527    |
| mmu00980  | Metabolism of xenobiotics by cytochrome P450 | 3               | 75     | 2.197296    |
| mmu00830  | Retinol metabolism                          | 3               | 78     | 2.150205    |
| mmu00320  | PPAR signaling pathway                      | 3               | 81     | 2.105092    |

"SelectionCounts" stands for the Count of the DE genes' entities directly associated with the listed PathwayID;  
"Count" stands for the count of the chosen background population genes' entities associated with the listed PathwayID;  
"Enrichment_Score" stands for the Enrichment Score value of the PathwayID, it equals $-\log10(P$ value$)$.

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expression could be induced by increasing the extracellular Ca$^{2+}$ concentration [40], calcium may be considered to suppress the epithelial-mesenchymal transition (EMT) to inhibit CRC metastases.

**Per3** was the most upregulated gene in our study, belonging to the period genes. Those genes are core elements of the transcriptional/translational feedback loops that generate the endogenous circadian rhythm, and **Per3** participates in timekeeping in the pituitary and lung [41]. Recent studies have suggested that circadian genes participate in the growth and development of various cancers, and the period genes have now been linked to DNA damage response pathways, inhibition of the growth of cancer cells, and increase in the apoptotic rate [42]. Oshima’s study has shown that the expression of **Per3** in CRC tissues was significantly lower than that in the adjacent normal mucosa, suggesting that **Per3** may function as a tumor suppressor gene [43].

**Tef** was the second most upregulated gene in our study. It belongs to the PAR-domain basic leucine zipper (PAR bZip) transcription factors and is involved in detoxification and drug metabolism [44] and in the maintenance of cell shape in fibroblasts [45]. It also serves as a pro-apoptotic gene by promoting the expression of bcl-gs or bik [46,47]. Many other genes were also upregulated, e.g., **Raf152** and **Prdx6**. **Raf152** is a novel RING finger protein and has been shown to have pro-apoptotic activity [48]. **Prdx6** is a member of the PRDX family, associated with functions such as cell proliferation, differentiation, and apoptosis, thus executing anti-cancer activity [49].

By using pathway analysis, we found that the Wnt pathway was significantly affected. Although the expression of the Wnt inhibitory factor **wif1** was significantly downregulated, that of many genes associated with the Wnt signaling pathway and their target genes was downregulated [i.e., **Tcf7**, **Myc**, **cyclin d1 (Ccnb1)**, and **Mmp7** at FC of −1.53, −1.61, −1.62, and −7.07, respectively]. The protein expression of β-catenin was significantly reduced in the DMH + Calcium group, suggesting that the Wnt pathway was downregulated in this group. This is consistent with previous findings [28,50]. Interestingly, the cell cycle pathway was also significantly inhibited in the DMH + Calcium group, with many genes in this pathway being consistently downregulated, i.e., the cyclins **Ccnb1** and **Ccnd1** were downregulated by 1.79 and 1.62 times, respectively, and the expression of the cyclin-dependent kinase **Cdki** was reduced by 1.66 times.

Among the metabolic pathways, the arachidonic acid pathways were significantly inhibited in the DMH + Calcium group. Many

![Figure 3. Expression of β-catenin in the non-tumor colorectal mucosa in the 4 groups determined by immunohistochemistry.](https://example.com/figure3.png)

| Table 4. Partial list of the hub genes. |
|----------------------------------------|
| **Name** | **Total No.of targeted genes** | **No.of overlap genes** | **Percent overlap** | **Gene set seed** | **P value** |
| Expression Targets of Jun/Fos | 542 | 68 | 12% | Jun/Fos | 1.55E-08 |
| Expression Targets of PKA | 369 | 52 | 14% | PKA | 2.03E-08 |
| Expression Targets of EGF | 504 | 64 | 12% | EGF | 2.71E-08 |
| Expression Targets of FOXM1 | 108 | 24 | 22% | FOXM1 | 4.04E-08 |
| Expression Targets of TPS3 | 538 | 65 | 12% | TPS3 | 1.41E-07 |
| Expression Targets of MYOCD | 36 | 13 | 35% | MYOCD | 1.66E-07 |
| Expression Targets of NF-kB | 742 | 76 | 10% | NF-kB | 8.28E-06 |
| Expression Targets of TNF | 900 | 87 | 9% | TNF | 1.69E-05 |

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enzymes in this pathway showed reduced expression, e.g., Ptgs2, also known as cyclooxygenase-2 (COX-2), was reduced by 3 times. The arachidonic acid pathway is activated during inflammation, leading to the synthesis of prostaglandin and thromboxane, which play an important role in inflammatory response [51]. Furthermore, the key enzyme in this pathway, Ptgs2 (COX-2), plays an important role in the initiation of CRC. COX-2 overexpression was shown to be correlated with carcinogenesis in more than 80% of CRCs [32]. In animal models, COX-2 expression was found to be sufficient to induce tumorigenesis [33]. The mechanism may be related to the activation of inducible nitric oxide synthase (iNOS) and VEGF signaling to promote formation of new blood vessels [54]. On the basis of the finding that the expression of the inflammatory cytokines S100a9, Defa, TNF, and TNF receptors was also significantly downregulated in the DMH+Calcium group, we speculated that calcium may play an important role in reducing inflammation-related tumorigenesis.

Among the Pathway Studio-derived hub genes, which may play key roles in the calcium-mediated prevention of CRC, FoxM1, NF-κB, etc., were also involved. FoxM1 belongs to a family of evolutionarily conserved transcriptional regulators that were characterized by the presence of a DNA-binding domain known as the forkhead box domain. Higher expression of FoxM1 was noted in many types of human cancers [55]. FoxM1 may play important roles in cell proliferation and apoptosis [56]. The molecular mechanism underlying FoxM1 signaling-mediated induction of tumor growth has not been completely elucidated. However, multiple oncogenic pathways such as PI3K/Akt, NF-κB, extracellular signal-regulated kinase (ERK), mitogen-activated protein kinase (MAPK), VEGF, reactive oxygen species (ROS), c-myc, and hypoxia-inducible factor (HIF)-1 have been reported to interact with FoxM1 signaling; this suggests that the interaction between FoxM1 signaling and other signaling pathways may play important roles in tumorigenesis [57]. It should be noted that the expression of many genes regulated by NF-κB showed altered expression in the DMH + Calcium group, e.g., TNF, PTGS2, MTC, CCND1, ETS-related gene 1 (ERG1), S100A9, and lymphoid enhancer factor 1 (LEF1), suggesting that NF-κB may also serve as a hub gene in the calcium-mediated prevention of CRC. Although the expression of NF-κB did not change significantly at the transcriptional level, it may have changed at the translational or posttranslational level. The expression pattern of NF-κB in the DMH + Calcium group and its specific mechanism needs to be further elucidated.

We also found that the gene expression levels of most of the above selected genes in mice from the DMH + Calcium group with tumors was between those from the same group without tumors and those from the DMH group, thus confirmed the importance of those genes in the calcium’s prevention of CRC. Interestingly, the expression of some calcium transporters, such as Trpv6, Trpv3 and PMCA, was different among the mice from the DMH + Calcium group and extracellular calcium, thereby activating the proliferation-related pathways [59]. Therefore, the differential expression of these calcium transporters disrupted the distribution of intracellular and extracellular calcium, thereby activating the proliferation-related pathways [59]. Hence, we speculated that the differential expression of these calcium transporters may partially account for the different expression levels of the selected genes.

It is necessary to mention the limitations of this study. First, we used colorectal tissues instead of colorectal mucosa in our microarray study. There are many different types of cells in the colorectal tissues, so it is hard to determine the cellular origin of the identified genes in this study. However, more and more evidences have shown that the stroma and immune cells play important roles in the tumor initiation. In our studies, we have found that there are many immune cells infiltrated into the colorectal tissues by pathological examination and the expression of many inflammation cytokines is significantly upregulated in the DMH group compared with Control group. These cells can not only cause local inflammation, but also significantly promote tumorigenesis [34]. We also have found that high calcium diet could reduce the expression of many inflammation cytokines, suggesting that calcium may play an important role in the prevention of inflammation-related tumorigenesis. Therefore we believe that using colon tissues instead of colon mucosa could reflect the overall and more realistic changes in

Figure 4. Potential hub genes derived from Pathway studio software. The differentially expressed genes regulated by the 2 hub genes (A,FoxM1/B, NF-κB) are displayed in form of a network diagram. Green or red coloring, sequences that are downregulated or upregulated in the DMH+Calcium group compared with the DMH group, respectively. The intensity of the color is proportional to the extent of change. doi:10.1371/journal.pone.0022566.g004
the gene expression. To our knowledge, colon tissues are widely used in the study of gene expression associated with CRC [60,61]. Second, since we did not use semi-purified diets in our animal experiment, our results may be biased. Third, the calcium content in our study was rather high. Although this may account for the drastic effectiveness in the prevention of CRC and no obvious adverse effect was found in the mice in our study, it is difficult to apply it for the prevention of sporadic CRC in humans, because exceedingly high calcium and vitamin D concentrations in the diet may pose a risk for developing hypercalcemia and kidney stones. However, a meta-analysis found that a calcium intake of more than 1000 mg/day will have a better preventive effect [62]. Another clinical trial found that a lower calcium intake may have no apparent preventive effect [10]. Therefore, further study is necessary to confirm the optimal calcium content in the prevention of CRC.

In conclusion, we confirmed the effectiveness of calcium to prevent CRC in the DMH-induced CRC mouse model. We also clarified the comprehensive mechanisms of calcium-mediated prevention of CRC. Our results showed that calcium exerted its protective effect mainly by downregulating the expression of oncogenes such as SI10ha9, Mmp10, Adam8, and Ptg2, by inhibiting the activity of tumor-associated pathways such as the Wnt and cell cycle pathway, and by negatively regulating cell proliferation, cell division, cell invasion, and angiogenesis. In particular, calcium is speculated to play important roles in reducing inflammation-associated tumors and suppressing tumor invasion and metastases. However, the mechanism of the calcium-mediated regulation of these genes remains to be studied.

Supporting Information

Table S1 Complete list of differentially expressed genes in the DMH group compared with the Control group. (XLS)

Table S2 Complete list of differentially expressed genes in the DMH + Calcium group compared with the DMH group. (XLS)

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