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High Sensitivity to Cholic Acid-induced Colonic Tumorigenesis Makes Female PIRC Rats (F344/NTac-Apc^{am1137}) a Suitable Model for Studying CRC-promoting Agents

CRISTINA LUCERI, ANGELO PIETRO FEMIA, MARIO D'AMBROSIO and GIOVANNA CADERNI

Section of Pharmacology and Toxicology, NEUROFARBA Department, University of Florence, Florence, Italy

Abstract. *Background/Aim: Rats of the adenomatous polyposis coli (Apc)-mutated female polyposis in rat (PIRC) (F344/NTac-Apc^{am1137}) model exhibit a low level of intestinal tumorigenesis and are thus potentially exploitable as a model for identifying substances increasing colorectal cancer (CRC). Materials and Methods: To test this possibility, we treated such rats with the bile acid (BA) cholic acid (CA) (0.3% w/w in the diet), known to promote CRC, and assessed tumorigenesis. Results: Precancerous colonic lesions (mucin-depleted foci) and intestinal tumors were dramatically increased in CA-treated rats compared to controls ($p<0.01$). Colon mucosa proliferation was higher and apoptosis lower than those in controls. Expression of nuclear receptor 1h4 (Nr1h4) gene [encoding for BA receptor farnesoid X receptor (FXR)], organic solute transporter beta (Ostb) and fatty acid-binding protein 6 (Fabp6), FXR-dependent BA transporters, were dramatically down-regulated in CA-treated rats. Conclusion: CA-increased tumorigenesis in female PIRC rats, with mechanisms involving increased proliferation, reduced apoptosis and marked down-regulation of genes controlling BA homeostasis. Since BAs have been implicated in CRC, we suggest that female PIRC rats can be used to identify CRC-promoting agents.*

The rat model polyposis in rat coli (PIRC) (F344/NTac-Apc^{am1137}), with a germ-line mutation in the adenomatous polyposis coli (*Apc*) gene, develops spontaneous tumors in the small intestine and in the colon, with mechanisms similar to those of human pathology (1). Indeed the *Apc* gene is considered the key gene of colorectal cancer (CRC), and the tumorigenesis in this rat strain arises as a multistep process, allowing the visualization of microscopic dysplastic lesions (*i.e.* mucin-depleted foci: MDF) that precede macroscopic tumor development and can be used as cancer biomarkers (2, 3). This model shows a strong gender effect, with male rats developing many more tumors than do females (4). Accordingly, studies aimed at the identification of dietary components or drugs with chemopreventive activity against colonic carcinogenesis, mostly used males, which provides a stronger statistical power when assessing a reducing effect on tumorigenesis (5, 6). On the other hand, female rats developing fewer intestinal tumors might be exploited for the identification of substances increasing carcinogenesis such as carcinogens present in the diet (or in toxicological studies), a possibility, to our knowledge, not tested to date.

Diets containing cholic acid (CA) have been used in experimental studies to mimic high-fat diets typical of Western countries and associated with high CRC risk (7-9). In fact, CA-enriched diets increase the intestinal concentration of deoxycholic acid (DCA), a well-known CRC promoter (10, 11) that stimulates intestinal carcinogenesis (12, 13).

Given these considerations, we thought it of interest to test whether and to what extent it would be possible to increase intestinal tumorigenesis in female PIRC rats by treating them with CA in their feed and determining microscopic MDF in the colon and intestinal tumors after 3 and 6 months, respectively. To study the possible mechanism of action of CA, not previously studied in this model, we determined cancer biomarkers such as proliferative activity and apoptosis in colon tissue and the expression of some genes involved in the process of carcinogenesis and in the homeostasis of bile acids (BAs). Furthermore, the association among these biomarkers was investigated with principal component analysis (PCA) and multiple regression analysis.

Materials and Methods

Animals and treatments. PIRC (F344/NTac-Apcam1137) and wild-type Fisher F344/NTac rats (originally obtained from Taconic,

Correspondence to: Giovanna Caderni, Section of Pharmacology and Toxicology, NEUROFARBA Department, University of Florence, 6 Viale Pieraccini, 50139 Florence, Italy. E-mail: giovanna.caderni@unifi.it

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Table I. *Primer sequences*.

Gene	Encoded protein	Forward Primer	Reverse primer	Base pairs
Actb	β-Actin	ACCACAGCTGAGAGGGAAATC	AGAGGTCTTTACGGATGTCAACG	277
Ccnd1	Cyclin D1	GCGTACCCTGACACCAATCT	CTCTTCGCACTTCTGCTCCT	180
Myc	Myelocytomatosis proto-oncogene	TCTGTGGAAAAGAGGCAACC	CTCATCTGCTTGAACGGACA	435
Bok	BCL2-related ovarian killer	TGGCCCAGGCTAAAGCACTA	CGATATACGCTGGGACGGAT	168
Bax	BCL2-associated X protein	CAACATGGAGCTGCAGAGGA	TGTCCAGCCCATGATGGTTC	205
Birc5	Baculoviral inhibitor of apoptosis repeat-containing 5	TGAGGAAGGGAGTGGATGAG	TCCATTACCCCATGGTAGGA	212
Muc2	Mucin 2	CCATCTCCACCACCATTACC	CAGATGAAGTCAGTGGGGAAG	435
Nr1h4	Nuclear receptor 1h4	GGAAGTGCAGAGAGATGGGA	AAGGAACATGGCCTCGACTG	437
Ostb	Organic solute transporter beta (solute carrier family 51b)	TCCGTTCAGAGGATGCAACT	TGGTGGGGCTTTGTCTAACC	407
Fabp6	Fatty acid-binding protein 6	GTTCAAGGCAACCGTGAAGATG	GATGGGTTGCAGTCCCTCAG	187

Taconic Farms, Inc. Rensselear, NY, USA) were bred at the University of Florence as described elsewhere (14). Pups were genotyped at 1 month of age (1, 14) and maintained in polyethylene cages under an experimental protocol approved by the Commission for Animal Experimentation of the Italian Ministry of Health (approval number: 323/2016-PR). Female PIRC rats aged 1 month were randomly assigned to a control AIN-76 diet (Piccioni, Gessate, Milan, Italy) given *ad libitum* (controls, n=7) or to the CA-treated group fed the same AIN-76 diet supplemented with cholic acid (Sigma-Aldrich Cheme, Darmstadt, Germany) at 0.3% w/w (n=7). The diet was fed for 3 or 6 months in order to evaluate the effect of CA at different times, reflecting early and late stages of tumorigenesis process, respectively.

Processing of the colon and collection of the samples. At sacrifice, the entire intestine (small intestine and colon) was dissected, flushed with cold saline and longitudinally opened to enumerate macroscopic tumors as described elsewhere (14). A segment of morphologically normal colon mucosa was scraped and stored in RNA-later™ (Qiagen, Milan, Italy) at –80˚C until analysis.

Determination of preneoplastic lesions, proliferative activity and apoptosis in the colon. MDF were then determined in the whole colon fixed in formalin for at least 24 h and stained with High-Iron Diamine Alcian blue (HID-AB) technique as described by Femia *et al*. (3). The colon was then embedded in paraffin to obtain histological longitudinal sections (4-μm-thick) and stained with hematoxylin-eosin to evaluate apoptosis and proliferation. Briefly, apoptosis was evaluated in at least 15 full longitudinal crypt sections by enumerating cells with the following characteristics of apoptosis: Cell shrinkage, loss of normal contact with the adjacent cells of the crypt, chromatin condensation or formation of round or oval nuclear fragments (‘apoptotic bodies’), as previously described (15). Proliferative activity was assessed in morphologically normal mucosa by determining proliferating cell nuclear antigen (PCNA) immunoreactivity as previously reported (14), studying at least 15 full longitudinally sectioned crypts.

Reverse transcriptase – polymerase chain reaction analyses. Total RNA was extracted from tissue homogenates with a commercially available kit, following the manufacturer’s instructions, including the DNase treatment step (Macherey-Nagel, Bethlehem, PA, USA). For first-strand cDNA synthesis, 100 ng of total RNA from each sample was reverse-transcribed with RevertAid RT Kit (Thermo Scientific, Waltham, MA USA). Primers were designed to span an exon–exon junction, on the basis of the rat GenBank sequences (Table I). PCR conditions were: 95˚C for 5 min and 30 cycles at 95˚C for 30 s, 60˚C for 30 s and 72˚C for 55 s. PCR products were separated on a 1.8% agarose gel and visualized using SafeViewTM (ABM Inc. Vancouver, Canada). Gel images were captured by a digital camera and the intensity of the bands was analyzed with Quantity-One software (Bio-Rad, Segrate, Milan, Italy). The expression of each gene was normalized to that of co-amplified beta-actin and reported as fold change compared to the mean expression of that gene in the control group.

Statistics. Data are presented as means±SE. Comparison between controls and CA-treated groups were performed using Student’s *t*-test. Comparisons among controls and CA-treated rats for 3 and 6 months were performed by two–way ANOVA and multiple range test using GraphPad Prism 7.0. Multiple linear regression analyses, including PCA, were performed using Statgraphics Centurion XVI software (The Plains, VA, USA). PCs having eigenvalues greater than 1 were retained and only factor loadings with an absolute value greater than 0.3 were interpreted. *p*-Values of 0.05 or less were considered statistically significant.

Results

CA-associated tumorigenesis. The weight of the animals at the beginning of the experiment was 62.4±3.9 g (n=14). Three months later, at the time of the first sacrifice, the body weight was 206±10.9 g and 204±2.4 g for controls (n=3) and CA-treated rats (n=4).

The determination of MDF in the colon after 3 months of feeding showed that the number of these precancerous lesions was much higher ($p<0.01$) in the group treated with CA compared with untreated controls (Figure 1A). The multiplicity of these lesions (number of crypts/MDF) was similar between the groups.

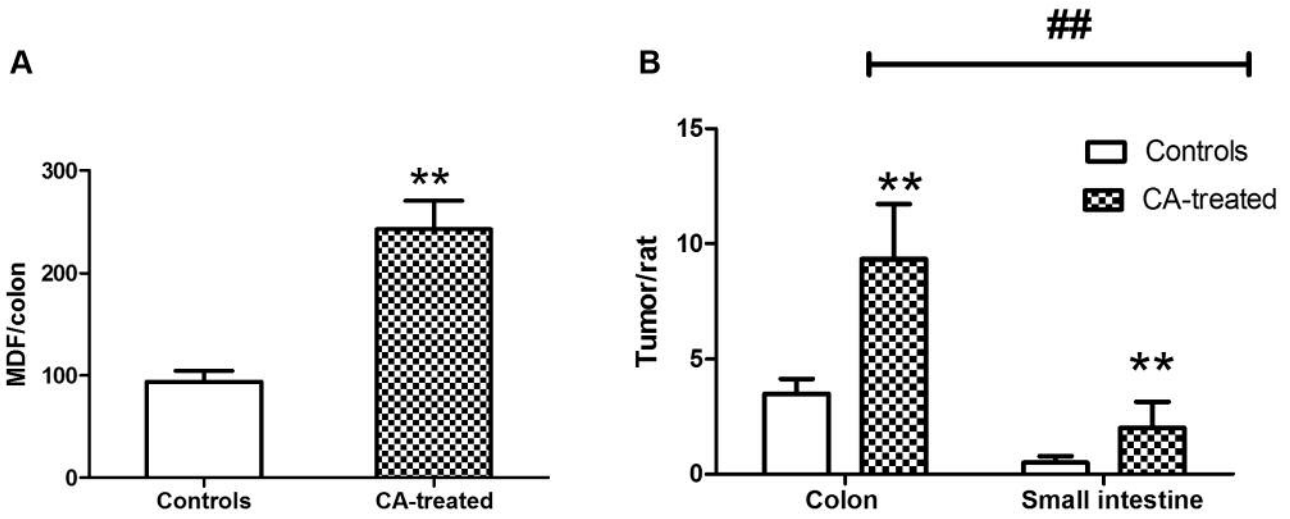

Figure 1. *Intestinal tumorigenesis in female PIRC rats fed with a control diet or with a diet supplemented with cholic acid (CA). A: Number of mucin-depleted foci (MDF)/colon. B: Number of tumors/rat determined in the colon and small intestine. Bars are mean values+SE. Significantly different at p<0.01 **CA-treated vs. controls; ##colonic vs. small intestinal tumors.*

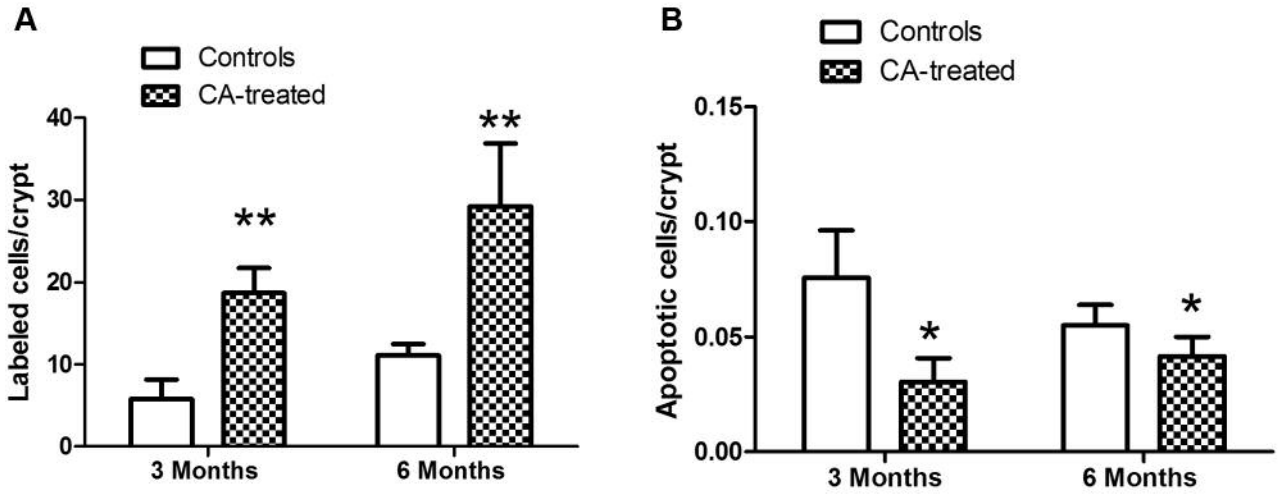

Figure 2. *Effects of cholic acid (CA) on colonic mucosa in female PIRC rats. Proliferative activity was evaluated as the number of proliferating cell nuclear antigen PCNA-labeled cells/crypts (A) and apoptosis as number of apoptotic cells/crypts (B) in rats fed for 3 or 6 months with a control diet or with a diet supplemented with CA. Bars are means+SE. Significantly different vs. controls at *p<0.05 and **p<0.01.*

To evaluate the effect of CA also at a later stage of tumorigenesis, one group of animals was sacrificed after 6 months of CA treatment. The number of macroscopic tumors along the intestine (in both the colon and the small intestine) in the CA-treated group was significantly higher ($p<0.01$) compared with controls (Figure 1B). We also noted that irrespective of the treatment, the number of colonic tumors was higher ($p<0.01$) in the colon than in the small intestine. The body weight of the animals remained similar (202±3.3 g and 205±2.9 g, in controls (n=4) and CA-treated rats (n=3), respectively (means±SE).

Proliferative activity and apoptosis in colonic mucosa. CA significantly increased cell proliferation as determined by PCNA expression in the crypt, an effect that was present both after 3 and 6 months of treatment (Figure 2A). The pattern of proliferation was similar between the two groups, with proliferative activity localized mainly at the bottom of

A

Gene name	Fold change CA *vs.* CTRL	*p*-Value
Ccnd1	1.82±0.06	0.050
Myc	2.73±0.09	0.003
Birc5	1.36±0.05	0.020
Bax	-1.84±0.13	0.004
Bok	-1.51±0.04	0.058
Muc2	1.30±0.02	0.075
Nri1h4	-24.83±3.91	0.001
Ostb	-1.54±0.09	0.045
Fabp6	-1.86±0.11	0.011

B

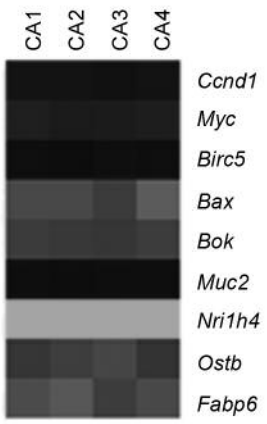

Figure 3. *Expression profiles of colonic mucosa of PIRC rats treated with cholic acid (CA) for 3 months. A: Fold change of expression of each gene of interest in response to CA treatment versus the mean expression of that gene in the control group (CTRL). *Student's t-test. B: Each column represents a different CA-treated rat, each row a different gene. The intensity of grey parallels the level of expression; dark grey indicates up-regulation, light grey indicates down-regulation compared to that in control rats.*

the crypt (data not shown). We also noted that older animals tended to exhibit higher proliferative activity than younger ones (Figure 2A), an effect that did not reach statistical significance (p=0.08). Apoptosis was slightly but significantly lower in the rats treated with CA at both time points (p<0.05) (Figure 2B).

Gene expression in the colon mucosa. We then determined the expression of some genes involved in carcinogenesis and in BA homeostasis by studying rats after 3 months of CA treatment in which the CRC-promotory effect of CA was already evident. The differences in gene expression between the two experimental groups were reported as fold changes (Figure 3A) and as a cluster (Figure 3B). The results showed that the expression of cyclin D1 (*Ccnd1*), myelocytomatosis proto-oncogene (*Myc*) and baculoviral inhibitor of apoptosis repeat-containing 5 (*Birc5*) (which also exerts pro-proliferative activity) was significantly increased in rats treated with CA. On the contrary, the expression of the pro-apoptotic genes BCL2-associated X protein (*Bax*), and BCL2 related ovarian killer (*Bok*) was reduced in CA-treated rats compared to controls. Expression of mucin 2 (*Muc2*), encoding for the most abundant mucin in the colon, was slightly but not significantly, up-regulated in rats treated with CA for 3 months. Interestingly, the expression of nuclear receptor 1h4 (*Nr1h4*) [the gene encoding for BA receptor farnesoid X receptor (FXR)] dramatically decreased in rats treated with CA, an effect that was further verified by determining the expression of two genes under *Nr1h4* control, fatty acid binding protein 6 (*Fabp6*) and organic solute transporter beta (*Ostb*). Indeed, these two genes were both significantly down-regulated in rats treated with CA compared to controls.

Table II. *Factor loadings for the parameters included in the principal component (PC) analysis. Loadings of ≥0.30 are shown in bold.*

	Component	
Factor	PC1	PC2
MDF, number	**0.331**	–0.013
PCNA-Iabeled cells/crypt	**0.319**	–0.004
Apoptotic cells/crypt	–0.196	**0.753**
Ccnd1	**0.341**	0.136
Myc	**0.343**	0.133
Birc	0.285	**0.376**
Bax	**–0.341**	0.091
Nr1h4	**–0.332**	0.266
Ostb	**–0.328**	0.151
Fabp6	**–0.315**	–0.390
Variance explained (%)	81.068	11.619
Cumulative variance explained (%)	**92.69**	

MDF: Mucin-depleted foci; PCNA: proliferating cell nuclear antigen; *Ccnd1*: cyclin D1; *Myc*: myelocytomatosis proto-oncogene; *Birc5*: baculoviral inhibitor of apoptosis repeat-containing 5; *Bax*: BCL2-associated X protein; *Nr1h4*: nuclear receptor *1h4*; *Ostb*: organic solute transporter beta; *Fabp6*: fatty acid-binding protein 6.

Multivariate analyses. PCA was used to assess combinations of biomarkers, rather than individual ones, with the aim of detecting meaningful associations among the various parameters determined in the present work. We observed that the first two principal components (PC1 and PC2) were able to explain 92.69% of the total variance (Table II): PC1 might represent variance associated with proliferation and CA-related signaling because these items had high loadings with opposing sign while PC2 might account for the remaining variance in the population not captured by PC1 and reflect mainly the apoptotic process.

The number of MDF was significantly and positively correlated with *Ccnd1* and *Myc* and inversely correlated with *Bax, Nr1h4, Ostb* and *Fabp6* gene expression. Multiple regression analysis identified a statistically significant relationship among the number of MDF/colon and the expression of *Cld1*, *Myc* and *Bax* genes ($p<0.0206$) and a borderline association among number of MDF, proliferative and apoptotic activities, and *Fabp6* and *Nr1h4* gene expression ($p=0.056$).

Discussion

The main result of this study is that a CA-containing diet doubled spontaneous tumorigenesis in female PIRC rats. Indeed, both the number of MDF and colonic tumors were significantly higher in CA-treated rats than in controls. Previous studies with PIRC rats documented that spontaneous carcinogenesis can also be increased administering dextran sodium sulphate, a synthetic polysaccharide acting as a strong inflammatory stimulus on the colonic mucosa (16, 17). Although dextran sodium sulphate is useful in studying the development of CRC associated with chronic inflammation (*e.g.* ulcerative colitis-associated risk of CRC), its strong toxic effects may interfere with the interpretation of results obtained. A previous study also documented that a high-fat diet enhanced benzo(a)pyrene-induced tumorigenesis in PIRC rats (18), although that same diet was not effective in enhancing carcinogenesis when tested alone. Here we showed that CA strongly enhanced intestinal tumorigenesis in PIRC rats. BAs have been implicated in human carcinogenesis since their level in the fecal stream is increased during the consumption of diets rich in lipids associated with increased risk of cancer (19), such as diets high in red meat and processed meat (20). Therefore, the present results suggest that these rats are highly sensitive to increased tumorigenesis induced by dietary promoters. In addition, the fact that such a high-fat mimicking diet increased tumorigenesis provides a stronger statistical power for also assessing the chemopreventive effects of drugs or dietary components in female PIRC rats, a possibility that could be explored in the future.

Regarding the mechanisms of action, we observed an increase in proliferative activity in the colonic mucosa, confirming mitogenic activity of BAs in the colon. Accordingly, rectal instillation of DCA produces a dose-related increase of proliferation in the colon of mice (21). Similarly, previous studies documented an increase in colonic cell proliferation in animals fed diets enriched with CAs (22, 23). On the contrary, a previous study feeding CA (0.2%) to F344 rats did not observe significant increase in proliferation but only a slight effect (12). We also observed that apoptosis was lower in CA-treated rats compared to controls, a result in agreement with Barone and coworkers, who studied apoptosis in the colonic mucosa of rats fed with BA-enriched diet (23).

As regards to gene expression, we found that *Myc* and *Ccnd1*, proto-oncogenes up-regulated in colonic carcinogenesis and down-regulated by chemopreventive treatments (6, 24, 25), were up-regulated in CA-treated rats. Similarly *Birc5*, also under Wnt signaling control (26) was slightly up-regulated in CA-fed rats. The increase in the expression of these pro-proliferative genes can be linked to the observed increase in proliferation as observed in the colonic mucosa with immunohistochemical experiments. Similarly, we observed down-regulation of two pro-apoptotic genes (*Bax* and *Bok*) (27) which may perhaps be linked to the observed lower level of apoptosis in the colonic mucosa. We also observed a slight increase in *Muc2* expression. MUC2 is the most abundant mucin secreted by goblet cells in the colonic mucosa (28). An increase in *Muc2* expression was previously documented by Song and colleagues in colonic cancer lines exposed to BAs (29), as well as by Lee *et al*. (30). Thus, the slight increase in *Muc2* gene transcription we found, might be interpreted as a response to the damaging insult to the cells lining the colonic lumen. In accordance with this, DCA has been shown to deplete mucin in goblet cells, thus perhaps causing damage (31); more recently, Wang *et al*. showed that CA-fed mice had a lower number of goblet cells expressing MUC2 compared with controls (13).

Nr1h4, is the gene encoding the BA FXR, which is expressed in the intestine (32, 33) and which has been reported to act as intestinal tumor suppressor (32–34). Accordingly, its expression was shown to decrease during colonic carcinogenesis in various experimental settings (33–35). Here we found that Nr1h4 expression was significantly down-regulated by CA treatment, an effect that was further substantiated by the concomitant down-regulation of two genes under *Nr1h4* control: *Fabp6* and *Ostb*. Previous studies also showed down-regulation of *Nr1h4* and *Ostb* in rodents fed a high-fat Western diet (36), indicating down-regulation of this receptor in conditions of increased BA concentration in the intestinal lumen.

In conclusion, we found that CA increased spontaneous carcinogenesis in female PIRC rats, with mechanisms associated with increased proliferation, reduced apoptosis and marked down-regulation of *Nr1h4*. Since BAs have been implicated in human carcinogenesis, these results suggest that

female PIRC rats can be used as a model to test the effect of diets suspected of promoting colonic carcinogenesis.

Conflicts of Interest

The Authors declare no conflicts of interests in regard to this study.

Authors' Contributions

APF carried out the carcinogenesis and the histochemistry experiments. CL together with MDA performed the gene expression analyses. GC determined apoptosis and proliferation in immunohistochemistry. GC, CL and APF conceived, designed and supervised the work, as well as drafted the manuscript, that was read and approved by all the authors.

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