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Molecular genetic evaluation of *Cichorium endivia* L. as an anticancer agent against colorectal cancer

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Abstract

Medicinal plants confer protective effects against a wide range of cancers including colon cancer. *Cichorium endivia* L. has many biological activities and functions, such as anti-inflammation and hepatoprotective effects. This activity is mainly due to its high levels of antioxidant compounds. The effect of cichorium extract and fluorouracil (5-FU) against colorectal cancer (CRC) induced by 1, 2- Dimethyl hydrazine (DMH) was examined in male albino mice. Where, different doses of cichorium extract (200, 400 and 600 mg/kg body weight) were investigated to determine the significant effective dose against colorectal cancer using differential display-PCR (DD-PCR), DNA sequencing, histopathological and cytological techniques. The results showed that the cichorium extract dose 200 mg/kg body weight was the best one compared to the others (400 and 600 mg/kg body weight). This finding could be a powerful therapeutic against colorectal cancer.

Keywords: Colorectal cancer - DMH - 5-FU - *Cichorium endivia* L. - DD-PCR

Introduction

Colorectal cancer (CRC) is a malignant tumor arising from the inner wall of the large intestine (the colon). Colorectal cancer (CRC) is the third most common cancer in both men and women and it is the second leading cause of cancer-related mortality in the developing countries due to the limited prevention and treatment options [1]. In Egypt, due to the changes of eating habits and frequent sit, the disease incidence of CRC is increasing by 10-12% of the total cancer diseases in Egypt (Oncology Center Report, Gharbia, Egypt, 2011). Therefore, the research and development of potent preventive and anti-cancer agents against CRC are very urgent. 1,2- Dimethyl hydrazine (DMH) is highly specific indirect colorectal carcinogens that induce the initiation and promotion steps of colorectal carcinogenesis yielding colorectal tumour lesions in a dose-dependent manner in rats, mice and hamsters [2]. Fluorouracil (5-FU) is a drug that is a pyrimidine analog which is used in the treatment of cancer. It is a suicide inhibitor and works through irreversible inhibition of thymidylate synthase. It belongs to the family of drugs called the antimetabolites. 5-FU has been given systemically
for anal, breast, colorectal, oesophageal, stomach, pancreatic and skin cancers [3]. About 25% of prescribed drugs in the world originate from plants and over 3000 species of plants have been reported to have anticancer properties [4]. *Cichorium endivia* L. is very valuable nutritionally, with a high content of dietary fibre, potassium and vitamin C. The natural components of this vegetable also have many biological activities and functions, such as anti-inflammation and hepatoprotective effects. This activity is mainly due to its high levels of antioxidant compounds [5]. The aim of this study is to investigate *Cichorium endivia* L. as an anticancer agent and an immune response drug especially on colorectal cancer (CRC). The aim of the current study is to investigate the therapeutic effect of *Cichorium endivia* L. against DMH-induced colorectal cancer in male albino mice using differential display-PCR (DD-PCR) and DNA sequencing. In addition, histopathological examination of colon tissues and analysis of chromosomal abnormalities in mice bone-marrow cells were investigated.

Materials and Methods

Cichorium endivia L. collection and preparation

Cichorium endivia L. plants were obtained from the crop research farm of the Crop Department, Faculty of Agriculture, Alexandria University, Egypt. The plant species were correctly identified by the Department of Botany, Faculty of Science, Alexandria University, Egypt. Plants were washed, dried and then ground using a blender [6]. One hundred gram of the leaves powder was soaked in one litter of double distilled water for 72 hours in shaking incubator (150 rpm/30 C). The sample filtrate was concentrated using rotary evaporator at 40 C, dried using lyophilizer, weighed and then stored until use.

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Experimental animal model

Thirty-two male albino mice (weighing about 25-30 g) were obtained from Medical Research Institute, Alexandria University, Egypt. Mice were examined for health status, housed and handled under ethical conditions according to the international rules of animal care. The animals were acclimatized for 4 weeks before the start of the experiment and they were given free access food and water. All animals were observed daily for abnormal signs. Thirty-two male albino mice were randomly divided into eight groups. Control group (n=4) mice didn't receive any treatment. DMH stopped group (n=4) mice were injected with 20 mg/kg body weight DMH twice per week for only three weeks [7]. DMH continued group (n=4) mice were injected with 20 mg/kg body weight DMH twice per week but continuously till the end of the experiment. 5-FU stopped group (n=4) mice were treated with DMH for three weeks then received 5-FU (80 mg/kg body weight) for two weeks then scarified. 5-FU continued group (n=4) mice were treated with DMH for three weeks then received 5-FU (80 mg/kg body weight) for two weeks then mice were kept without any treatment till the end of the experiment. *Cichorium* 200 (Ch 200) group (n=4) mice were treated by DMH for three weeks twice per week then treated with 200 mg/kg body weight/day for 40 days. *Cichorium* 400 (Ch 400) group (n=4) mice were treated by DMH for three weeks twice per week then treated with 400 mg/kg body weight/day for 40 days. *Cichorium* 600 (Ch 600) group (n=4) mice were treated by DMH for three weeks twice per week then treated with 600 mg/kg body weight/day for 40 days. All animals, except those of 5-FU stopped group, were sacrificed after 9 weeks from the beginning of the experiment. At the end of the experiment (24 hours after the last dose of the *Cichorium* treatment), the mice were killed under proper ether anesthesia after an overnight fast. The present work was performed on both blood serum and colon tissue. Blood was collected in EDTA coated tubes for complete analysis, centrifuged at 2000xg for 10 min to separate plasma and stored at -80ºC until analysis. The colon tissue was immediately removed and washed twice with ice-cold saline solution.

Synthesize of First-strand cDNA

RNA was extracted from blood serum and colon tissue samples using RNzol LS Reagent according to the manufacturer's protocol. RNA isolated was reverse transcribed into cDNA in a total volume of 25 µl using High Capacity cDNA Reverse Transcriptase Kits. The Reverse transcription from RNA to cDNA was performed using 4 µl of RNA per sample, 2.5 µl 10x RT buffer enzyme, 5.5 µl oligo d(t) primer, 2.5 µl 25x dNTPs Mix (100 mM), 1 µl MultiScribe™ Reverse Transcriptase (50 U/µl and 5.3 µl Nuclease free water. Amplification was performed in a programmable thermal controller (PTC-100TM) thermocycler. The amplification protocol was 25 C for 5 min followed by 1 hr at 42 C and finished with denaturation step at 70 C for 15 min.

Differential display-PCR (DD-PCR)

Differential display was performed in a 25 µl volume according to [8]. The reaction was carried out using four arbitrary primers (A2: 5'-GAAACGGGTGGTGATCGC-3' [9], A4: 5'-GGACTGGAGTGTGATCGC-3', AT1: 5'-ATTTCCTTGAAGAGAACGGTGC-3' and AT2: 5'-AGTTCGGCCAGCATCTGCTCGT-3'). Reaction was performed using 12 µl PCR master mix (promega), 5 µl Nuclease free water, 8 µl primer, and 1 µl cDNA template. Amplification was performed in a Quanta Biotech Thermocycler and the PCR reaction conditions was 94 C for 5 min for one cycle, followed by 92 C for 30 sec, 35 C for 2 min, 72 C for 90 sec for 5 cycles and finished with 92 C for 30 sec, 40 C for 30 sec, 72 C for 90 sec, 72 C for 5 min for 35 cycles. DD-PCR products were resolved by electrophoresis in 1.5% agarose gel with 0.5X TBE buffer. The resultant PCR product was excised from the gel and purified using a MEGAquick-spin™ (INtRON) total fragment DNA purification kit. The purified DNA was stored at -20 C till use in DNA sequencing.

DNA sequencing

The purified DNA was sequenced using the automated sequencer of Sanger method by Macrogene Company (Korea). DNA homology searches were carried out with the National Center for Biotechnology Information (NCBI) databases, using the basic local alignment search tool (BLAST) network service.

Histopathological studies

Colon tissues were fixed in 10% formalin for 24 hour, washed with running water, dehydrated in ascending grades of alcohol (70%, 80%, 95% and absolute alcohol) and cleaned by immersion in xylene followed by impregnation in melted paraffin wax in oven at 60 C for 1 hr. The specimens were embedded in paraffin and were left to solidify at room temperature. Using a rotary microtone, sections of 5 µm thick were cut and were mounted on clean glass slides. Finally, the samples were stained with conventional hematoxylin and eosin (H&E) stain for examination under light microscope for any histopathological changes.

Analysis of chromosomal abnormalities in mice bone-marrow cells

Mice used in the present study were cytogenetically healthy. Three hours prior to killing, the animals were injected with 0.6 mg/kg of colchicines. After killing, the adhering soft tissue and epiphyses of both tibiae were removed. The marrow was aspirated from the bone, transferred to phosphate buffered saline, centrifuged at 1000 rpm for 5 min and the pellet re-suspended in 0.075 M KCl. Centrifugation was repeated and the pellet was re-suspended in fixative (methanol:acetic acid, 3:1). The fixative was changed after 2 hrs and the cells suspension was left overnight at 4 C. Cells in fixative were dropped onto very clean glass slides and air-dried. Spreads were stained with 10% Giemsa at pH 6.8 for 5 min. Slides

were coded and scored for chromosomal aberrations such as gaps, deletion, fragment, break, stickiness and polyploidy. A mitotic index based on at least 200 cells was recorded. For chromosomal abnormalities at least 200 scorable metaphase cells per dose were recorded.

Results

The up and down regulated genes in colorectal cancer

Differential display PCR was carried out using four arbitrary primers (A2, A4, AT1 and AT2). The results indicate that there are more than 247 band patterns obtained for scanning the cDNA in colon cancer mice tissues. Selection of the up and down regulated genes was dependent on the absence or presence of the bands in the control group, respectively. However, the results revealed that only 7 out of the 247 bands were unique (up or down regulated genes). Two up-regulated genes with different lengths (700 and 200 bp) were observed with primer A2. Primer A4 showed one up-regulated gene (450 bp) only. Primer AT1 gave two up-regulated genes (300 and 350 bp). Primer AT2 gave two regulated genes; one was up-regulated gene (200 bp) and the other was down-regulated gene with molecular weight 190 bp (Figure 1). These seven bands were excised from the gel, purified and sequenced. DNA nucleotide sequences of these genes were aligned with the other published mice genes using the BLAST software at NCBI server (www.ncbi.nlm.nih.gov/BLAST). Sequence alignments were carried out using *Mus musculus* Translated BLAST (blastx) online program. The cDNA nucleotide sequences were illustrated in Table 1.

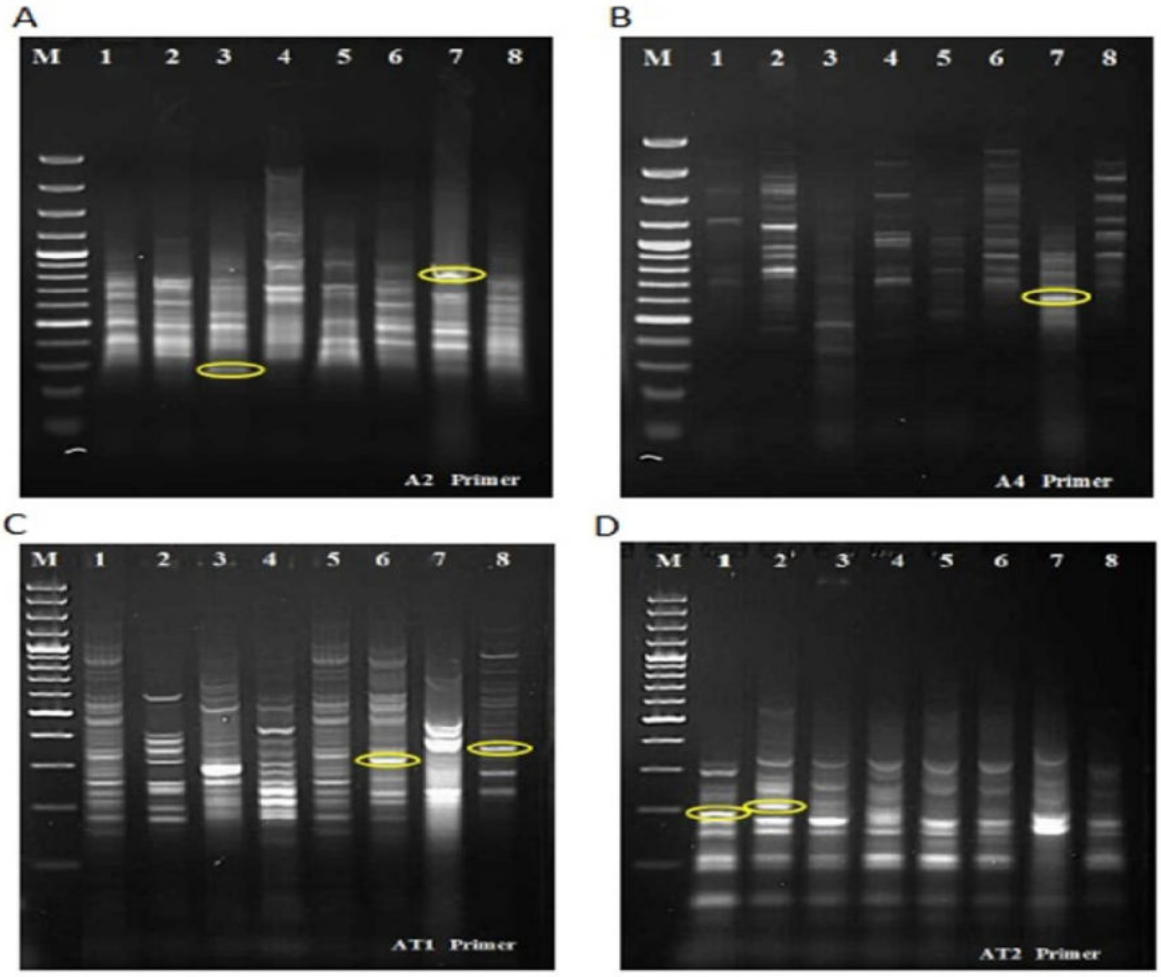

Figure 1. Differential display using A2, A4, AT1 and AT2 primers. (A) A2 primer, (B) A4 primer, (C) AT1 primer and (D) AT2 primer. Lane M: 1300 bp ladder DNA marker, lane 1: control group, lane 2: DMH stopped group, lane 3: DMH continued group, lane 4: 5-FU stopped group, lane 5: 5-FU continued group, lane 6: Ch 200 group, lane 7: Ch 400 group and lane 8: Ch 600 group.

Table 1. Differential display of PCR sequence results and the identified genes.

Primers	Molecular Weight	Lanes	Sequence	Expected gene	Ident
Primer A2	200	3	CGC TGG ACT GCG CAG GGT GCC AGA CCT CAT CCA TTC CTG ACT TGG CCC GGG GCT CCC GGC CCA GCT GCT CCN ACA CCC CAT TTA TGA CCA TGA GAC CAG CTT CCT CAC GGG TTC CAC GAG TCA TGT NCT CNC ANG GTG GAG GTC TGA CAT CCT NNN CAA CCA TGC GTC CAC TTG CTG CTA CTG CAG CTG CTG GAC CCA CTC CAG CTG TCG CCA CCG CTCT	Poly A binding protein	56%
	700	7	CCT TGC NGA CCN TTG GCG CCC AGA ACT GAA TGG NCT CCT CAC CAA TGA GAC CGT GTC TGA CCC CGA TTT CAN TGG ANT GTT GGA TCG ANT ACA CAT TCT GAC CGC CGT TNC CTT TAT CGC CAA AAA CGG TGA GGA TTC CCG CTT GCC GGC GCG ACT CCT GNN GTT CTT TGT GGC TCA GCA AGT CGC CGG TCT GAA TGT GTA TNT GAT CTC ACT CGC GNG TGG TGG AAC CGT CAN NNN NGT CTC GAT CGT CTT TAC TGC CAT TAG TTT GTC NNC GAC ATG NGT CAG GCC GGC CGN ATG NNN TGN TNA NGC AAA TGA TCT TGN TTT NNC TAC TTN NTN NGG GCA CCG TTN TNN NAT GGN ANN AAT NNN NAN NGG GAC NGG CGT TNN CTC CGN ACC CGT TGT TGG TCC ACT CCA CGT AAA TGT CCT CGN GCNTGA NNC TGT NAC CAT GCA GGT CAN AGT GAC CTG TTT CTT AGT NTN CTC TTC TTC TGG NGG AGG CAA NAC ATA TAC CTG TGG AGC TCT TAC TGA CCC TGN GAG GGT ANA ANC NTA CAC AAA GGT CAT GGC TGA AGN NGA NAG CTG TCN	Immunoglobulin gamma-2a heavy chain	93%
Primer A4	450	7	GCN CGT GCT CAT TCC NGN NGC AGC TGC TCG TTA TAA TGA TGA TCT GCC CTG CNT GNG AAC CTC CTT TTC CAG CCC ACC CCC TCC TCA TGG NGG CCA CAT GGG TGG AGG CGA GAC CGG AGA GGC GAN NAT AAN AAT GCG GGC CAN ATA ANN ANN NAT TAA CAG GTT CNC ANN NGN ATG AGN TTT GGC AGA TGT GGG GGA GTA TAA CGC TCG ATA TTG GNC CAT TAG NNN TAG GNC NCN AGC AAA TAC NAN NGC GAT NTA TCC NNT CAN NCC CGG NCC CGG TCC CAN NTA AAG AAN TAT NGN	Sp110 nuclear body protein-like	39%
Primer AT1	300	6	GGT CTC GAT GTC CCC GAA CTC TTG TCC AGG CTC CTG CCC CTC GCC CCC TTG TTT TTT TCA CTG CTG ATC TCA CTA GGT GCA CTT TCC AAT CCC GAA TCC CTC GCG GNN CTT CGT GAC CTC NAT GTN NAA TTC GCC CAT GGA TGA GGA CGA TGA TGC CTT GAG GAA GTC TGG GGA CAC TGC CAA AGA CGA TTA ATA TTA TGT GTG GAA CTT GAA TTT GGC CAA CGC GCC TCG CCTG	Ephrin type-B receptor 6	27%
	350	8	TGT NAC ACC TGT TTT GTT CGA CTC CCA ACT TGG GTT ACT GTG ACC CTG NCG CCT GGA TGT GTG TGA CGC ATG AGC TTA AAC TAG AAG ACG CGC GGC GTG GGA CCC CCT TCT CCT GCT GNC TAC AGG NGN GTT GCA TTT CAC ACG ATC TCC TGT GAA NCG CTT CGN TAC TTC ACC NCN NGC CCA AGC TCA CCT CCA GGA ATC CAC AGT GTG GNG AAT NCA AAT AAG AGG AGG TTC TGT CTT TTT TCC CGG TTN GCT GCN CAA CGA TCG GTT GAG TGC GAT CCT GAG CAC CGG TCC CTG CGG A	Sodium- and chloride-dependent GABA transporter 2 isoform X2	52%
Primer AT2	200	1	ACT CNG TNN TCN TCC ACT TTG GAT TGT TTG NGA CCC GTG ACG CTT CTC TCC CCG TCA GAA TTC CTG CCG CTC TTT AAT GGT TAT TGC CTG TTA GGC GGC GCG CTA ACA CTT ATC TAT GAA CCA TTT CNN AAA AAC ATG AAA TAA AAT GGA CTG CTG ACG CAA ACA AAT GAA ATT TTC CAT ACG TCT GAT AGT ATA ACA CTC ATT ATT CAC CCC TTA TTT C	Cullin-associated NEDD8-dissociated protein 1	37%
	190	2	CTC NNN GCG ANC TCG CTG CTT TGN NTT GTG CGG NCG TCN CGA CGC AAC GGT CCG NTG NNG CCA TGG CTT CGN NNC NTC NCG GGA NTC NAC CCG TCA TCT CGG CCT GNT GTA GGC NCC GCN NCT TCT CCC GAG CAN ATG NTG NNC AAA TTC TNA CAN T	Coiled-coil domain containing 162 isoform X7	50%

Histopatological studies

Investigation of the control group showed normal colon tissues, whereas, the cancerous group showed well differentiated colorectal cancer with complete loss of cellular architecture and high degree of cellular proliferation (hyperplasia). Histopathology examination of 5-FU groups showed moderate differentiated CRC, lytic necrosis and portal inflammation. Also the histopathological examination of Ch groups showed repair and healing in the tissues as well as hyperplasia (Figure 3).

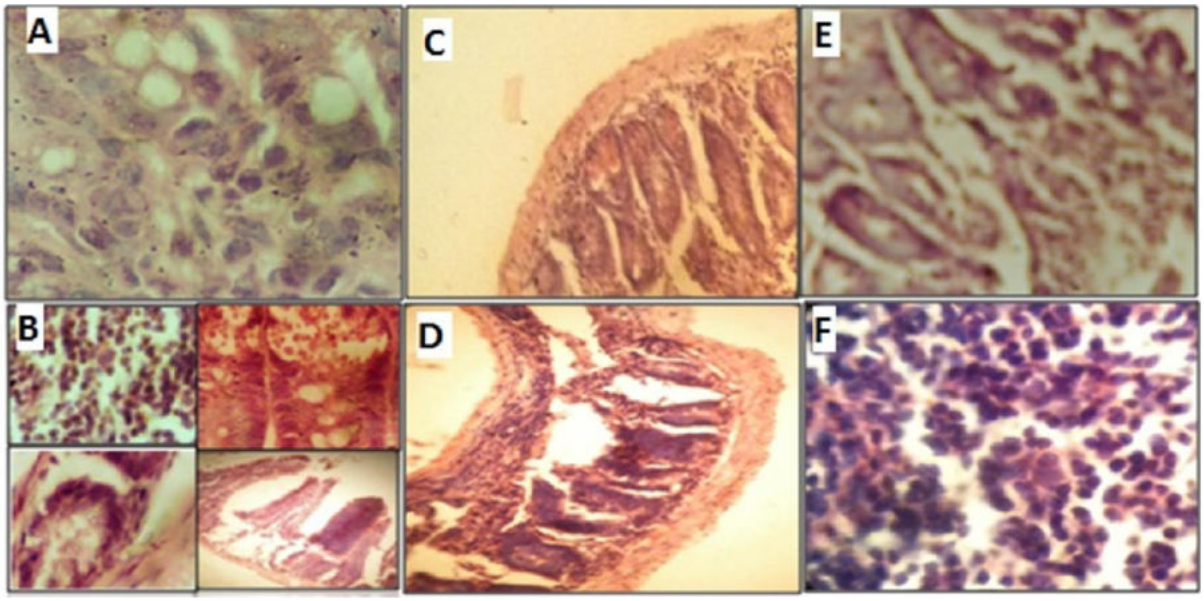

Figure 3. Histopathological studies in colonal tissues. (A) Normal colonal tissues in control group, (B) Colonal tissues showing hyperplasia, initiation of cancerous cells, cancerous cells and high damage in DMH group, (C) Colonal tissues treated with 5-FU, (D) Colonal tissues treated with Ch 200, (E) Colonal tissues treated with Ch 400 and (F) Colonal tissues with hyperplasia condition in Ch 600 group.

Cytogenetic studies

Cytogenetical examination showed that there were different types of abnormalities (gap, deletion, polyploidy, Robertsonian Centric Fusion (RCF) and stickiness) in DMH groups (mice with CRC). However, the main type of the observed aberration was stickiness (Figure 4). The percentages of aberrations ranged from 3% in Ch to 5% in 5-FU groups. Table 2 shows the data obtained from the cytological examination of mice-bone marrow cells obtained from the different treatments. Mitotic index (MI) was calculated according to the following formula: MI%= (Number of dividing cells/Total examined cells) x 100.

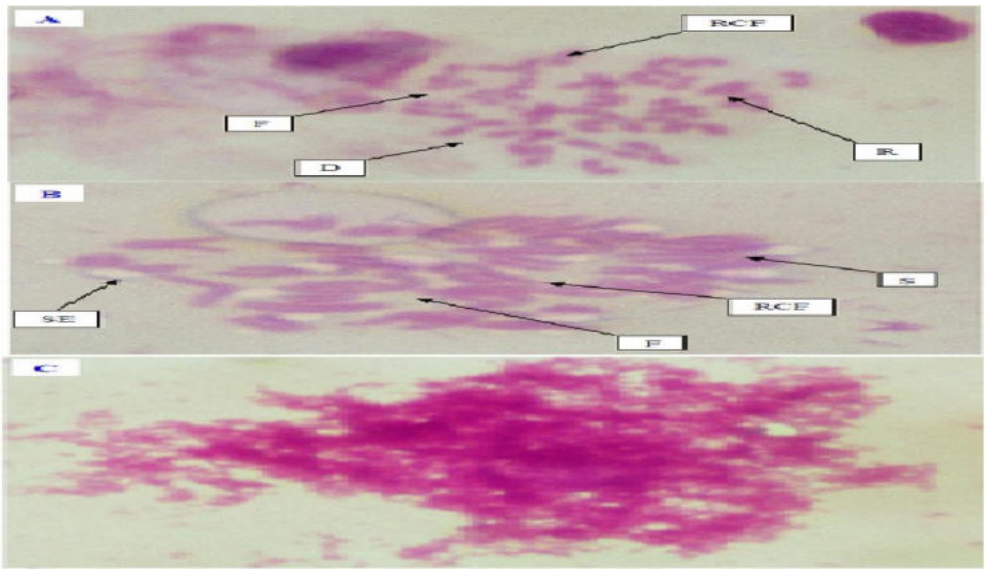

Figure 4. Photomicrograph showing metaphase stage in mice bone marrow cells with types of aberrations. (A) Metaphase stage with Ring chromosome (R), Chromatid Deletion (D), Robertsonion Centric Fusion (RCF) and Fragment (F). (B) Metaphase stage with Robertsonion Centric Fusion (RCF), Fragment (F), Stickiness (S) and Sticky Ends (SE). (C) Metaphase stage with Poly ploid cells.

Table 2. Mitotic indices (MI) and the percentages of chromosome aberrations examined in 200 metaphase cells.

Groups	MI	Stickiness	RCF	Fragments	Deletion	polyploidy	Total
Control	14.2	3	-	1	-	-	4
DMH stopped	1.3	8	4	2	3	5	22
DMH continued	2.5	4	2	1	1	1	9
5FU stopped	4.1	2	1	2	-	-	5
5FU continued	5.3	3	-	1	1	-	5
Ch. 200	8.1	2	-	1	2	-	5
Ch.400	10.2	2	-	-	1	-	3
Ch.600	11.4	3	-	-	1	-	4

Discussion

In the current study, differential display PCR and sequence analysis techniques were used to determine and recognized the selected up and down regulated genes using four primers (A2, A4, AT1 and AT2). Where, primer A2 showed two up-regulated genes, the first one was poly (A)-binding protein (PABP) which detected in the DMH continued group. [10] observed that the expression of PABP was significantly correlated with disease-free survival and overall survival in patients with stage II and stage III colorectal cancer. PABP expression was positively associated with survival outcome, and may have predictive value in the prognosis of patients with colorectal cancer. Their findings indicated that PABP may play a role in the pathogenesis of colorectal cancer. The second up-regulated gene (Immunoglobulin Gamma-2a heavy chain) was associated with Ch 400 group. Immunoglobulin Gamma (IgG) was identified in prostate cancer cells. The siRNA targeted silencing of immunoglobulin heavy constant gamma (IGHG) can inhibit cell viability and promote apoptosis, which might therefore act as a potential target in prostate cancer gene therapy [11,12]]. Primer A4 showed one up-regulated gene (sp110 nuclear body protein-like) only in Ch 400 group. This gene acts as a transcription factor and plays a role in the innate immunity against intracellular pathogens and promotes apoptosis of infected cells (Pan et al., 2005). AT1 primer revealed that two up-regulated genes were in Ch 200 and Ch 600 groups. The first gene from AT1 primer (Ch 200 group) was Ephrin type-B receptor 6, which is a kinase-defective receptor for members of the ephrin-B family (B1 and B2). [13] recognized that Ephrin type-B receptor 6 modulates cell adhesion and migration. Elevated levels of expression and activity have been correlated with the growth of solid tumors, with Eph receptors of both classes A and B being over expressed in a wide range of cancers including colon cancer [14]. The other gene from AT1 primer (Ch 600 group) was sodium- and chloride-dependent GABA transporter 2 isoform X2. There is evidence that γ-Amino butyric acid (GABA) receptors play a role in the proliferation of tumor cells developing in the brain, prostate and liver [15]. Finally, primer AT2 showed one up-regulated gene (coiled-coil domain containing 162 isoform X7) in DMH stopped group. Many coiled coil-type proteins are involved in important biological functions such as the regulation of gene expression such as transcription factors [16]. This means that one of those transcription factors which should be blocked was opened by using DMH. While, the other down-regulated gene (Cullin-associated neddylation (NEDD) 8-dissociated protein 1 (CAND1)) occurred only in the control group. [17] highlighted a novel role of the regulatory protein CAND1 in centriole duplication control and provided the first evidence of its deregulation in a considerable proportion of primary prostate adenocarcinomas.

Eventually, the different doses (200, 400 and 600 mg/kg body weight) of the cichorium extract showed that the dose 200 mg/kg body weight was the best one compared to the others (400 and 600 mg/kg body weight). The histopathological data and the cytogenetical examinations proved that DMH is a positive carcinogen. Hyperplasia as well as cancerous cells was observed in colon after treatment with DMH. Treatment with 5-FU after cancer induction the damage was decreased. Healing was observed, or at least no more damage, when doses of *Cichorium* 200, 400 and 600 mg/kg body weight were applied. However, the dose 200 mg/kg body weight of the *Cichorium* was found to be more affective in healing and disappearances of hyperplasia than the other doses. In a previous study, [18] found that *Cichorium endivia* L. has a significant effective anticancer in breast cancer cell line (MCF7). Also in another study, *Cichorium endivia* L. (200 mg/kg body weight) represented or showed an effective anticancer and immune response drug especially for colon cancer [19].

Conflicts of interests

The authors declare no conflicts of interests.

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