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The Efficiency of Resveratrol on Adenomatous Polyposis Coli Gene in Mice Colorectal Cancer Treated with Azoxymethane

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Abstract--- *Colorectal cancer (CRC) is the third most common cancer and the fourth most common cause of cancer-related death. Most cases of CRC are detected in Western countries, with its incidence increasing year by year. The probability of suffering from colorectal cancer is about 4%–5% and the risk for developing CRC is associated with personal features or habits such as age, chronic disease history and lifestyle. 5-Fluorouracil (5-FU) is the most commonly used therapy and the conventional treatment for colorectal cancer. However, its toxicity to normal tissues is considered a major obstacle to successful cancer chemotherapy; thus, resveratrol is used in combination with 5-FU to reduce its toxicity. The goal of our study is to investigate the mechanisms underlying the effects of resveratrol alone and in combination with 5-FU in inhibiting experimentally induced colon cancer. Gene expression analysis showed seemed lower expression of APC (adenomatus polyposis coli) gene in (+) control significantly difference between positive and negative control by given Azoxymethane and normal saline respectively, The mean expression levels of (-) control (n=6) and combination (Res. +5-FU) (n=6) mice in the colon, There are high statistically significant expression differences. Error bars represent the 95% confidence intervals.*

Keywords--- *Azoxymethane, Efficiency of Resveratrol, Polyposis Coli.*

I. INTRODUCTION

Cancer is a group of diseases in which genetically altered cells abnormally transform and autonomously proliferate. These cells are irresponsive to normal physiological stimuli, evade the immune system, invade nearby tissue and metastasize to distant organs. In carcinogenesis, malignant transformation of the cell occurs in three stages, initiation, promotion, and progression (Hanahan and Weinberg, 2011).

Tumour is a complex multi-stage biological process caused by many etiological factors. Throughout this process a normal cell obtains capabilities that cause its transformation into a tumourigenic, and may in the end become malignant cell (cancer) if the cell is not recognized and/or eradicated by the immune system (Huda Hameed Kadhim Alabbody and Inam Jasim Lafta2 2019)

Initiation is the earliest step in carcinogenesis that occurs due to reversible genetic alterations putting healthy cells at risk of subsequent malignant transformation. Although genetically mutated, initiated cells are not neoplastic and are morphologically identical to normal cells. Genetic mutation may stay dormant in the affected cell for years or may induce cell proliferation in autonomous and clonal patterns. In other words, the proliferation of the initiated

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cell transmits this phenomenon to daughter cells where the alteration becomes permanent and remains symmetrical by producing two newly initiated cells (Clancy, 2008;)

Cells have developed several mechanisms to identify and repair different forms of DNA damage caused by exogenous or endogenous Genotoxic carcinogens or replication errors. During the cell cycle, checkpoint mechanisms aim to ensure that DNA is undamaged before allowing DNA replication and cell division. Failures in these checkpoints can lead to DNA damage accumulation and eventually permanent alteration of the DNA sequence.

Colorectal Cancer

Colorectal cancer (CRC) is the third most commonly diagnosed cancer worldwide and ranks second among cancer-related deaths in many countries (Lee *et al.*, 2014). The proportions of newly diagnosed CRC cases are 9.2% in men and 10% in women, which accounts for 9.7% of overall cancer incidence worldwide (Dušek *et al.*, 2015). The risk of CRC increases with age, hereditary genetic predisposition, chronic bowel inflammation, and diabetes. In addition, smoking, alcohol consumption, low vegetable intake, obesity, and sedentary lifestyle are associated with an increased incidence of CRC (Rasool *et al.*, 2014). In contrast, chronic intake of certain on-steroidal anti-inflammatory drugs, high calcium consumption, and post-menopausal hormone usage are associated with reduced risk of the disease (Steward and Brown, 2013.).About 70% of CRC occurs without familial or inherited predisposition (sporadic disease), while less than 10% of patients inherit the tendency to develop the disease (Hasanpour *et al.*, 2014). The most common inherited conditions that predispose to CRC are familialadenomatouspolyposis (FAP) and hereditary nonpolyposis colorectal cancer (HNPCC, Lynch syndrome). FAP accounts for less than 1% of all CRC cases and is associated with autosomal dominant inheritance of a mutant adenomatous polyposis coli (*Apc*) gene. FAP patients develop hundreds of colonic adenomatous polyps that start malignant transformation when the patient is as young as 20 and almost all patients develop cancer by the age of 40 years unless the colon is removed. HNPCC is an autosomal dominant disease that accounts for 2-3% of all CRC cases. It is

Adenomatus Polyposis Coli (Apc) Gene

Mutations in *Apc* represent the key alteration found in the majority of CRCs. This earliest event in tumour initiation induces Wntsignalling due to inactivation of the *Apc* gene (located on 5q21), and is noticed in ~70% of sporadic adenomas. The *Apc* product, a 312 kDa protein, contains multiple functional domains that regulate differentiation, adhesion, polarity, migration, apoptosis, and chromosomal segregation (Aoki and Taketo, 2007). One of the most important issues in studies of APC is to identify the cellular pathways responsible for tumor genesis when it is mutated. APC is a multi-domain protein that contains binding sites for numerous proteins, including microtubules, the Wnt/Wg pathway components -catenin and axin, the cytoskeletal regulators EB1 and IQGAP1, and the Rac guanine-nucleotide-exchange factor (GEF) Asef1. Most (~60%) cancer-linked APC mutations occur in a region referred to as the mutation cluster region and result in C-terminal truncation of the protein (Beroud and Soussi, 1996). Because these truncations cause loss of the domains required for binding to -catenin and to

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microtubules the interaction of APC with -catenin or microtubules has been considered to be essential for its tumor suppressor activity. -Catenin has a dual role in cells, functioning both in cell.

Resveratrol

Resveratrol (3,5,4'-trihydroxy-*trans*-stilbene) (Figure 1), is a polyphenolic natural product which belongs to the group of phytoalexins that are synthesized when plants are subject to environmental stress, such as exposure to pathogens. It can be found in widely consumed foods and beverages such as red wine, grapes, peanuts, pistachios, berries, and It adori tea. In fact, resveratrol is produced by more than 70 species of plants in response to stress. Resveratrol is particularly thought to mediate some of the potential health benefits of red wine (Mishra, 2011). The effects of resveratrol appear to be achieved through numerous cell signalling pathways, including induction of cell cycle arrest, stimulation of apoptosis and differentiation, suppression of inflammation and angiogenesis, and reduction of adhesion, invasion, and metastasis (Storniolo and Moreno, 2012). Another recent study revealed that resveratrol prevents tumour growth by down regulation of the *Kr as* signalling pathway, using a genetically engineered mouse model, that mimics sporadic CRC with a knocked out *Apc* locus and activated *Kras* (*APCCKO/Krasmut*)(Saud *et al.,* 2014; Hung *et al.,* 2010). Mice received daily resveratrol at doses of 150 or 300pp (equivalent to 105 and 210 mg daily human dose, respectively) before colonic tumour formation. In this prevention study, 60% of mice did not form tumours while the rest revealed loss of *Kras* expression. In mice that were allowed to form colonic tumours before resveratrol treatment, there\ was a 33% complete remission rate and in the remaining animals, there was a 97% reduction in tumour size (Saud *et al.,* 2014).

5-Fluorouracil

5-Fluorouracil (5-FU) is used antitumor medicine for the first time in1957 especially in treatment breast tumors, colon tumors also tumors in neck and head (Grem J.L.2000).5-FU is a heterocyclic aromatic organic compound with a structure similar to that of the pyrimidine molecules of DNA and RNA; it is an analog of uracil with a fluorine atom at the C-5 position in place of hydrogen. Only one crystal structure is reported in the literature for pure 5-FU, in which the compound crystallizes with four molecules in the asymmetric unit and the molecule adopts a hydrogen-bonded sheet structure [Hulme A.T., et al 2005]. 5-FU reacted with nucleoside during the metabolism leads to cell death and cytotoxicity (Noordhuis P.2004).The overall response rate for advanced colorectal cancer of 5-FU alone is still only10–15% (Giacchetti S et al 2000), and the combination of 5-FU with other anti-tumor drugs have merely improved the response rates to 40–50% (Douillard J.Y.et al 2000). Therefore, new methods for treatment very important. Non- response to the treatment by using 5-FU is called treatment resistance. Recently, continuous investigation in the work of these molecules, that help in the chemotherapy.

Mechanisms of Action

5-FU is transferred to fluorodeoxyuridine monophosphate, which inhibits deoxythymidine mono-phosphate formation. dTMP is important for DNA division then at the end leads to cytotoxicity [Longley D.B.et al 2003]. Dihydropyrimidine dehydrogenase used as a cofactor to transfer 5-FU to dihydrofluorouracil in tumor and normal cells. More than (80) % of 5-FU is destroyed DPD in the hepatic tissue (Hsieh Y.F., *et al* 2008).

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Future Perspective

5-FU is very important in the treatment of cancer, but the response rate for the treatment of 5-FU only is low. so, finding the best treatment strategies leads to an increase insensitivity of 5-FU and resistance reverse to the drug (Tian, Z. Y.; *et al* 2007).

II. MATERIALS AND METHODS

Preparation of Animals

All thirty adult male *balbe*/C mice were acclimated for 3 weeks before the experiment and categorized in five groups as shown in Diagram (3-1).

The Grouping of the Animals and Experimental Procedure

On the day of the experiment:

A) G1 (6 mice) Control (-ve) gave normal saline

B) G2 (6 mice) Control (+ve) gave Azoxymthane 10mg/kg bw once weekly for two weeks

C) G3 (6 mice) Give Azoxymthane 10mg/kg bw once weekly for two weeks then Give resarveratrol 50mg/kg/bw. For 30 days

D) G4 (6 mice) Give Azoxymthane 10mg/kg bw once weekly for two weeks then 5-flurouracil (12.5)mg/kg. We gave i.p. injections 1 xweekly, for 4 weeks

E) G5 (6 mice) Give Azoxymthane 10mg/kg bw once weekly for two weeks then Give resarveratrol 50mg/kg/bw orally and gave 5-FU (12.5)mg/kg gave ip. Injection as combination for 30 days.

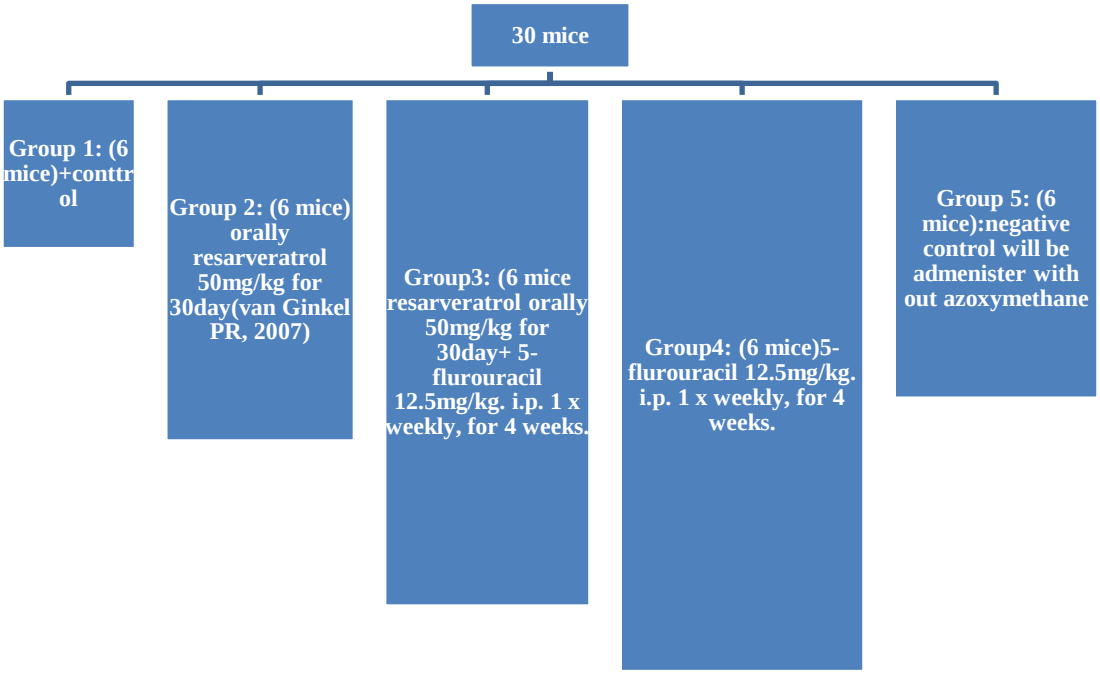

Diagram 3-1

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Gene Expression of Tumor Suppressor geneAdenomatus Polyposis coli (APC gene) and (B2m gene) by RT-PCR

Sample Preparation

Six mice per each group 30 mice in total were sacrificed and sampled. For genomic DNA and total RNA extractions, the mucosa (6 x 4 mm) was separated from the underlying sub mucosa and musculature under a dissecting microscope. Samples for RNA extraction were stored in RNA later uses Trizol for the isolation RNA. Trizol is a mixture of guanidine thioacyanate and phenol, which effectively dissolves DNA, RNA and protein on homogenization or lysis of tissue sample Gene.

Gene Expression Experiment

The expression of B2m and APC geneswere determined by using real-time reverse transcriptase PCR (RT-PCR). About 30 mg of colon tissues that were collected from the different groups of mice and kept in a Trizol-based RNA maintaining buffer solution at -20 before RNA extraction. The RNA extraction was performed according to the manufacturer's instructions using RNA extraction kit (abm, Canada). Total RNA concentration was determined using the Nanodrop (Thermofisher, USA). Real-time RT-PCRs were performed and pre-analysed by using the Agilent AriaMx Real-Time PCR System (Agilent, USA).

Real-time RT-PCR experiments were performed using the One-Step Bright Green qRT-PCR kit (abm, Canada). Samples were run in triplicate and contained 50 ng of RNA per reaction.

The initial step of One-Step Bright Green qRT-PCR was a one cycle reverse transcription reaction to generate a cDNA of the expressed mRNA of APC and B2m genes (42 °C for 15 minutes). Next step was a one cycle of a hot start reaction (95°C for 10 minutes) followed by the final amplification step (40 cycles of 95 °C for 15 seconds and 60°C for 60 seconds respectively (Figure 3.4). The primers that were used to amplify and quantify the expression of APC and B2m genes are listed in Table (1).

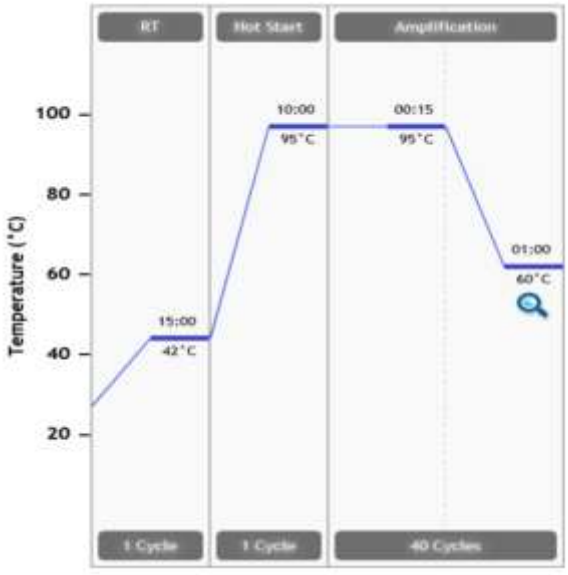

Figure 3: One-Step BrightGreenq RT-PCR Programmed Setting

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Virtually all individual results were within 0.5 cycle threshold units of the average triplicate value. Controls run without reverse transcriptase confirmed the absence of contaminating DNA in any of the samples.

The expression of mRNA of APC gene was normalised to that of the housekeeping gene B2m. The B2mgene has been demonstrated to be consistently expressed in mice (Matsuzaki et al., 2015). Normalised expression of the APC gene was calibrated against corresponding mRNA expression ofB2m gene. The results are given as the relative expression of the APC mRNA compared to that of control positive mice group.

In order to confirm that a nonspecific amplification did not occur, products were resolved on a 1% agarose gel and stained using ethidium bromide. No additional PCR products were noted for any of the reactions.

Table 1: Primers Used for Amplification and Quantification of the Expression of APC and B2m Genes

Gene	Primer name	5'-3'	Size of product	Accession number	Reference
APC	APC-F	GTGGACTGTGAGATGTATGGGC	150	NM_007462	Origene
	APC-R	CACAAGTGCTCTCATGCAGCCT			Origene
B2m	B2m-F	ACAGTTCCACCCGCCTCACATT	105	NM_009735	Origene
	B2m-R	TAGAAAGACCAGTCCTTGCTGAAG			Origene

III. Expression Profiles Results

The general expression levels for the colon were determined by expression measurements and calculating the mean value for those. These were then compared between and within treated groups.

First of all, compared the positive control group given AOM and NEGATIVE CONTRROL control mice in order to investigate whether the carcinoma status affected the overall gene expression levels in the colon. Figure (4) showed seemed lower expression OF APC gene in (+) control significantly difference between positive and negative control by given Azoxymethane and normal saline respectively

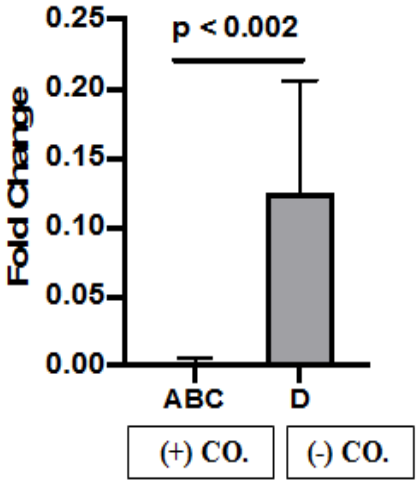

Figure 4: Showed Extremely Lower of APC Gene Expression in (+) Control (AZO.) When Compared with (-) Control. Gene Expression in Carcinoma and Negative Control Mice

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The mean expression levels of (-) control (n=6) and combination (Res. +5-FU) (n=6) mice in the colon tissue. There are statistically significant expression differences at $p \leq 0.05$. Error bars represent the 95% confidence intervals Figure(5).

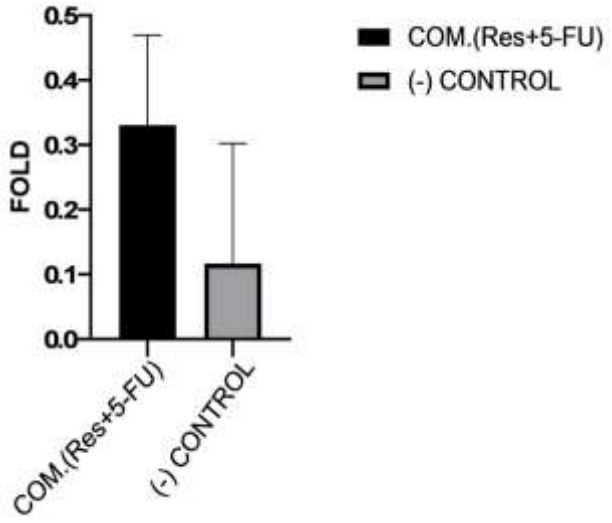

Figure 5: Compared the APC Gene Expression by RT-PCR between Combination and Negative Control

The expression levels were observed to be somewhat between 5-FU treated group and combination group that seemed to observed the combination group had highly expression level than 5-FU but no significant difference statistically at $p \leq 0.05$. However, the differences were not clear enough reach statistical significance between (5-FU and combination).Mean expression levels of (+) control mice samples were compared to investigate whether there were APC Gene expression levelwith 5-FU group and combination group treatedshowed differences significantly $p \leq 0.05$expression level than other groups, (Fig. (6).

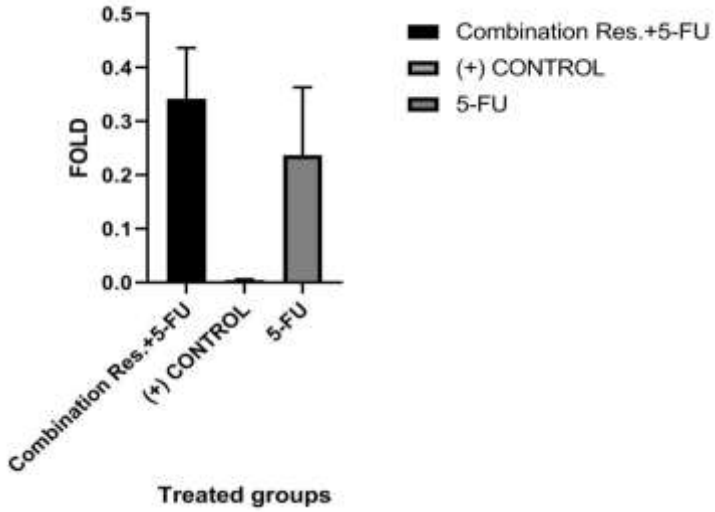

Figure 6: Showed the Fold Change of APC Gene Expression between Combination, (+) Control and 5-FU

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Figure (7) determined the resveratrol expression level effect at p 0.05 that have significant differences when compared with positive control group but had no significant differences when compared with 5-FU group treated.

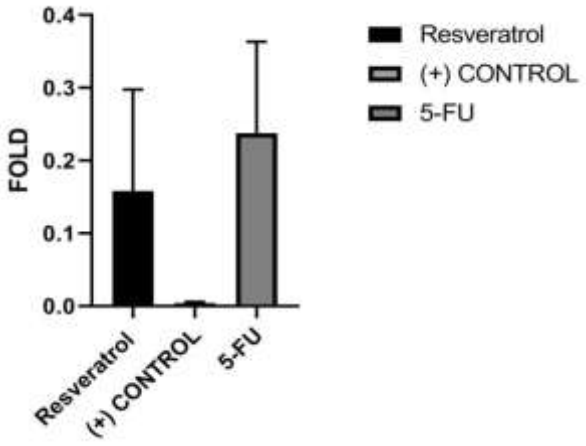

Figure 7: Representative the Change Fold of Expression Level between Three Groups (Resveratrol, Positive Control and 5-FU) Treated

Statistical Analysis

All data of gene expression analysis were expressed as mean ± standard deviation. Data were analyzed using tow -way ANOVA using prism program version 8**.**

IV. DISCUSSION

APC GENE Expression Discussion

In the current study, p53 was significantly increase in treated group 3 and 4 compared to colon cancer group (2). This result may be attributed to several mechanisms by which 5-FU can activate p53, such as incorporation of fluorouridine triphosphate into RNA, incorporation of fluorodeoxyuridine triphosphate into DNA and inhibition of TS by fluorodeoxyuridine monophosphate (FdUMP) with resultant DNA damage. Both TS inhibition and mis-incorporation of 5-FU metabolites

into RNA result in p53 stabilization (Longley et al. 2003). Also, resveratrol induced activation of p53 in colon tissue (fig.7). Resveratrol modulates phosphorylation of p53 at Ser 15, resulting in p53 stabilization and increasing its functional activity. In accordance with ourresults, Sato *et al*. (2013) found that the level of p53 phosphorylation at Ser 15, together with its transcriptional target p21, was increased after resveratrol treatment. Wu *et al.* (2004) also suggested that resveratrol can induce the S phase arrest of cancer cells and enhance the anti-tumoreffect of 5-FU on murine hepatoma22 as well as antagonizing its toxicity. Resveratrol biochemically modulates and enhances the therapeutic effects of 5-FU, may be potentially useful in cancer chemotherapy. Another mechanism was documented by (Chung *et al*. 2018) who provided the first evidence that resveratrol has the ability to enhance anti-telomeric and pro-apoptotic potentials of 5-FU in colorectal cancer, hence leading to resensitization to chemotherapy.

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