

Assessment of antioxidant status in different genotypes/phenotypes at codon 72 of TP53 gene for patients with sporadic colorectal cancer in Babylon province

Moaed E. Al-Gazally^{1*}, Alaa S. Al-Awad², Hamzah H. Kzar³

¹Department of biochemistry, Faculty of medicine, Babylon university, Iraq

²Oncology centre, Merjan teaching hospital, Babylon, Iraq

³Department of chemistry, Faculty of veterinary medicine, Al-Qasim green university, Iraq

Abstract: One of most frequent malignant disease in the developed countries is colorectal cancer and it is the seventh most common cancers among Babylon population /Iraq. It was reported that oxidative stress status is being play a very important roles in development of carcinogenesis. The purpose from this study was assessment of antioxidant status in different genotypes/phenotypes(GG/AA, GC/AP, and CC/PP)of patients with sporadic colorectal cancer in Babylon province. This investigate was done by using colorimetric method to measure of total antioxidant capacity(TAO-C) in patients with these genotypes/phenotypes. All patients included in this study were receiving adjuvant chemotherapy regimen and subdivided into two groups according to Duke's classification of malignant into early stages (A+B) and(C+D) advance stages, cancer site (colon and rectum), and according to the number of dosage of chemotherapy regimen (half dosage and total dosage). Colorimetric method was using to measurement of serum TAO-C ,while PCR-RFLP was used for TP53 gene codon 72 polymorphism analysis. The results were showed significant decrease in the mean-/+SD of serum TAO-C concentration of GG/AA genotypes/phenotypes in advanced stages of patients group compared to same genotypes/phenotypes in early stages.

Key words: Antioxidant status, TP53 gene, Sporadic colorectal cancer, Adjuvant chemotherapy.

Introduction

Colorectal cancer (CRC) is one of most frequent malignant and lethal diseases in the development countries. CRC is the general term using to described the cancers that occurred in the colon and rectum. It is one of the major cause of morbidity and mortality in the world^[1,2]. Also CRC is a disease that emanating from the epithelial cells that lining of the colon and rectum^[3]. The expanding of colorectal cancer either as a result from hereditary cancer syndromes, or sporadic, or inducing by inflammatory bowel diseases. Sporadic CRC forms 90% of patients with cancer of colon and rectum and the remaining 10% of patients having a family history of CRC^[4]. Western culture caused of increasing in the incidence of sporadic CRC in developed countries^[5]. The developing countries accounts for over 63% of all cases of colorectal cancer^[6]. Iraqi population started to converted to developing countries by shifting towards the western- lifestyle that has probability leads to increasing of the colon and rectal cancer incidence^[7]. In 2010, Iraqi cancer registry team was reported that CRC is the seven most common cancers in Babylon province^[8]. One of the most important

factors that associated with increased risk of CRC are life style and dietary components^[9]. Some of the epidemiological studies were showed that systematically high intake of dietary fats, red meats and proteins is positively related to the increase risk of CRC^[10]. CRC is currently treated with a chemotherapy regimen that is based on 5-fluorouracil (5-FU), or its oral prodrug analogue (capecitabine) in combination with oxaliplatin or irinotecan and response rates (RR) did not exceeded 40-50%. In general, patients with CRC who are not treated have a median survival of 5-6 months. With the development of the first chemotherapeutic agent, 5-fluorouracil (5-FU), the median survival was extended to 11-12 months^[11].

An imbalance between prooxidants and antioxidants in the cells caused by oxidative stress which is manifested by increased levels of free radicals^[12]. The defense system against oxidative stress is depending on the adequacy amounts of antioxidants that are derived either directly or indirectly from the diet^[14]. Substances that present at low concentrations compared with that of an oxidized substrate, is called antioxidants that inhibits oxidation of this substrate^[15]. Antioxidants including compounds that have a non-enzymatic and enzymatic nature^[16]. The TP53 gene encoding P53 protein, has a common sequence polymorphism that results in either Pro or Arg at amino acid position 72^[17]. The alleles of the polymorphism in codon 72, exon 4, encoded an Arg amino acid (CGC/ Arg72) with a positive-charged basic side chain and a Pro residue (CCC/ Pro72) with a non-polar aliphatic side chain. The Polymorphism of TP53 gene Arg72Pro occurs in Pro rich domain of P53(residues 64-92) of P53 protein, which needed by the protein to induce apoptosis^[18]. Several studies was showed the alterations of TP53 gene in many regions among world and no data available among Iraqi populations. According to available data, this is the first study showing a possible combined association of genetic polymorphic variants of the TP53 gene with sporadic CRC risk in Babylon province/ Iraq. The present study analyzed the polymorphisms Arg72Pro of apoptosis-related gene (TP53) and their impact on the response to adjuvant chemotherapy regimen of patients with sporadic CRC in Babylon population.

2-Materials and methods

This study was performed at the laboratories of Biochemistry Department, College of Medicine, University of Babylon. The collection of samples was conducted during the period from 1st of March 2014 till 30th of June 2015. The patients group who subjected in this study were (52) patients in the age group ranging from 39-75 years, the mean \pm standard deviation (SD) was (59.3 \pm 10.68 years). This group comprised of males (58%), with their age ranging from 39-75 years old, the mean \pm SD was (61.8 \pm 11.4 years), and females (42%) with age ranging from 39-73 years, and mean \pm SD was (63.1 \pm 11.3 years). The ages of patients group <50 years old were 22(42%) and at \geq 50 years old were 30 (58%).

All of those patients were screened and treated with adjuvant chemotherapy in the oncology centre of Merjan Teaching Hospital in Babylon province with clinical symptoms of colorectal cancer. The diagnosis of colorectal cancer were performed by Sigmoidoscopy, colonoscopy or CT- scan and clinical diagnosis was confirmed in all patients by histological examination. Fifty two apparently healthy individuals(without gastrointestinal diseases) were taken as a control group with the age ranging from 37-75 years, the mean \pm SD was (57.8 \pm 11.1 years). This group comprised of males (61%) their age ranging from 39 -73 years, mean \pm SD was (60.4 \pm 10.2 years), and females (39%) their age ranging from 37-75 years, mean \pm SD was (64.5 \pm 11.6 years).

The age and sex of this group were matched to age and sex of patient group, where statistical analysis showed non-significant differences in the age and sex between patient and control groups ($p > 0.05$). Each person who contributed in the control group underwent full history and physical examination including: address, age, gender, smoking, education, dwelling, past history of diseases and medications.

Determination of Total Antioxidant Capacity (TAO-C)

Total antioxidant capacity TAO-C was measured depending on FRAP (Ferric Reducing Antioxidant Power) colorimetric assay. At low pH, reduction of ferric tripyridyltriazine (Fe³⁺ -TPTZ) complex to ferrous form (Fe²⁺-TPTZ), that can be monitored by measuring the change in absorbance at 520 nm.

Tp53 gene codon 72 analysis

The AccuPrep[®] Genomic DNA Mini Kit(Bioneer, Korea) provides an efficient method for purifying total DNA from whole blood and frozen blood. Chaotropic salt was used to lyses cells and degrade protein,

allowing DNA to bind to the glass fiber matrix of the spin column. Contaminants were removed by using a Washing Buffer (containing ethanol) and the purified genomic DNA was eluted by a low salt Elution buffer or TE buffer. The presence of DNA extracted by the previous procedure was detected by using agarose gel electrophoresis technique. The extracted DNA was colorless, so a tracking dye (bromophenol blue) was used with DNA to ease the loading step of the electrophoresis procedure.

Amplification of exon 4 codon 72 of TP53 gene was done by polymerase chain reaction (PCR). Amplification was performed in a programmable thermal cycler gradient PCR system. The forward and reverse primers were: 5'-GCTCTTTTCACCCATCTACAG-3' and 5'- TGAAGTTCATGGAAGCCAGC-3', respectively. PCR-restriction fragment length polymorphism was used to investigate the polymorphism in exon 4 codon 72 of TP53 gene in control and colorectal cancer patients involved in this study.

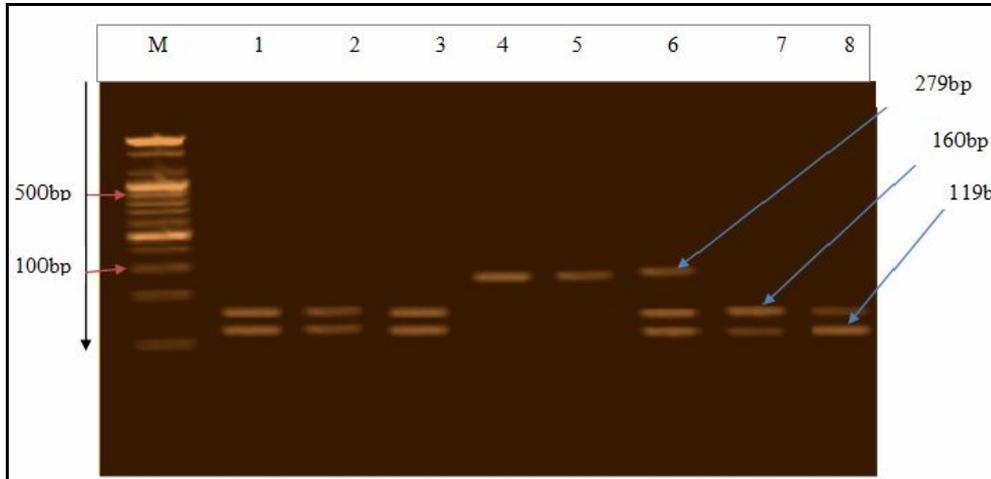
Optimization of PCR-RFLP conditions was done by method that described by^[19]:

- Different volume of primer (1 μ l, and 1.5 μ l).
- Gradient annealing temperature and choosing the conditions that gave best result. A master premix of Bioneer[®] was used to PCR process.

For exon 4 codon 72 of TP53 polymorphism, the PCR product was digested with the restriction enzyme *MvnI* (*Methanococcus vanniellii*) (EURX[®]-Poland) (restriction endonuclease) (*MvnI*+buffer \neq 1) .

Final volume of reaction was 50 μ l; In first buffer 5 μ l, PCR product 10 μ l, ddH₂O 34 μ l was added and then 1 μ l of working enzyme solution , mixed and incubated at 37 C^o for 1-3 hours and the time not affect on result (some of result obtained at incubation to overnight).

The uncut fragment was 279 bp (homozygote GG (Arginine allele)) , digestion products were 160 and 119 bp (homozygote CC (Proline allele)),and three bands (279,160,and 119) (heterozygote GC (Arginine/Proline)),as shown in fig. (1).



Figure(1): Electrophoretic picture represents the codon 72 of TP53 genotyping , where lane M is 100 bp DNA ladder , lane (4,5) have a single band at 279 bp representing the homozygous of (CC)Pro/Pro, lane 6 has a band at 279,160,and 119 bp representing heterozygous (GC)Arg/Pro allele ,and lane (1,2,3,7,8) has two bands at 160 and 119 bp representing the homozygous (GG)Arg/Arg.

Results

Clinic-pathological characteristics of patients with colorectal cancer whose included in this study were classified into two groups depending on (age, gender, chemotherapy treatment status, Dukes' stages of cancer, tumor location, smoking status, obesity status, , dwelling, and education status) (Table 1).

Table-1: Clinic-pathological characteristics of patients with sporadic CRC included in this study

| CLINICO-PATHOLOGICAL VARIABLES | NO. TOTAL=52 | PERCENTAGE (%) |
|---------------------------------------|---------------------|-----------------------|
| - Age | | |
| - <50 | 22 | 42 |
| - ≥50 | 30 | 58 |
| - gender | | |
| - Male | 30 | 58 |
| - Female | 22 | 42 |
| - Dukes' stages | | |
| - A+B | 19 | 36.5 |
| - C+D | 33 | 63.5 |
| - Cancer site | | |
| - Colon | 37 | 71.1 |
| - Rectum | 15 | 28.9 |
| - Chemotherapy status | | |
| - Total dosage | 30 | 57.6 |
| - Half dosage | 22 | 42.4 |

All subjects are categorized depending on the process of fragmentation of amplicon of TP53 gene exon 4 codon 72 being Arg/Arg(AA)(GG genotype) for homozygous polymorphism, Arg/Pro(AP)(GC genotype) heterozygous, and Pro/Pro(PP)(C/C genotype) homozygous, the comparison between G and C allele frequency (case-control) and evaluated a recessive model of G allele(CC vs. GG+CG) and a dominant model of G allele (GG vs. CC+CG), as shown in table(2).

Table-2: Genotyping/Phenotyping of TP53 exon 4 codon 72 polymorphism and allele frequency

| Group | Genotype/phenotype | | | Total | Allele frequency | |
|----------------|---------------------------|--------------|--------------|--------------|-------------------------|------------|
| | GG/AA | GC/AP | PP/CC | | G/A | C/P |
| Control | 10 (19%) | 36 (69%) | 6 (12%) | 52 | 54% | 46% |
| Patient | 22 (42%) | 25 (52%) | 5 (6%) | 52 | 66% | 34% |
| Total | 32 | 61 | 11 | 104 | | |
| p-value | S | NS | NS | | | |

The results of the present study showed the frequency of GG/AA genotype/phenotype in patients with sporadic CRC was more than in control group (42 vs 19%)(OR = 3.08, CI_{95%} (1.27-7.44). Also the present study suggests no statistical difference (P>0.05) in GC/AP and CC/PP genotypes/phenotypes in patients group compared to controls. The results showed that frequency of Arg allele was (0.66)(0.34) in patients and control group respectively, and found significant difference between Arg and Pro alleles in patients and controls (OR= 3.76), CI_{95%} (2.09-6.76) as shown in table(3).

Table-3 :TP53 gene polymorphism characterization in sporadic CRC patients and control group.

| GENOTYPE/PHENOTYPE | PATIENT | CONTROL | ODD RATIO | CI_{95%}** |
|---------------------------|----------------|----------------|------------------|---------------------------|
| GG/AA | 22 (42%) | 10 (19%) | 3.08* | 1.27-7.44 |
| GC/AP | 25 (48%) | 36 (69%) | 0.415 | 0.18-0.92 |
| CC/PP | 5 (10%) | 6 (12%) | 0.815 | 0.23-2.85 |
| G/A | 66% | 52% | 1.79* | 2.02-6.76 |
| C/P | 34% | 48% | | |

* Significant difference (P < 0.05).

** CI_{95%} : confidence interval at 95 % level .

The results in table (4) showed that there were a significant decrease in the mean-/+SD of serum TAO-C concentration of GG/AA genotypes/phenotypes in advanced stages of patients group compared to same genotypes/phenotypes in early stages.

Table-4: Comparison of mean-/+ SD of TAO-C patients with sporadic CRC with different genotyping/phenotyping in early and advanced stages.

| Genotype/Phenotype | TAO-C(U/ml) level in patients group(A+B) (Mean \pm SD) | TAO-C(U/ml) level in patients group(C+D) (Mean \pm SD) |
|--------------------|---|---|
| GG/AA n=22 | 11.2 \pm 2.6 | 9.2 \pm 3* |
| GC/AP n=25 | 11.2 \pm 3.7 | 10.9 \pm 3.2 |
| CC/PP n=5 | 9.9 \pm 4.5 | 10.1 \pm 3.7 |

*significant at p-value <0.05

** highly significant at p-value <0.001

Discussion

Colorectal cancer is one of the most common cancers in worldwide. It was reported that the highest incidence rates of CRC occurred in western countries. Sporadic CRC is caused by different factors such as environmental conditions, lifestyle, dietary habits, obesity, smoking, and lowest physical activity^[20]. Colorectal cancer is a heterogeneous disease with different molecular pathways leading to different phenotypes. Sporadic colorectal cancer is a multi-factorial disease arising from interaction between genetic background and environmental factors, such as diet or lifestyle, however, the exact role of the genetic background to sporadic CRC remains unclear. Genetic and epigenetic alterations act to dysregulate conserved signaling pathways involved in cellular metabolism, proliferation, differentiation, survival, and apoptosis^[21]. It was well known that the distribution of TP53 codon 72 polymorphism change in different geographic areas and ethnicities. Emerging evidence has shown that p53 gene participates in human carcinogenesis as tumor suppressors. Polymorphism of p53 gene Arg72Pro may influence the function of p53 protein and then affect the processing of carcinogenesis. It has been suggested that alterations of p53Arg72Pro was associated with genetic mutation or allelic polymorphisms in many human cancers, such as lung, breast, cervical, and colorectal cancer^[22]. Several studies showed the alterations of TP53 gene in many regions among world and no data available among Iraqi populations. According to available data, this is the first study showing a possible combined association of genetic polymorphic variants of the TP53 gene with sporadic CRC risk in Babylon province/ Iraq. The present study analyzed the polymorphisms Arg72Pro of apoptosis-related gene (TP53) and their impact on the response to adjuvant chemotherapy regimen of patients with sporadic CRC in Babylon population.

The results of the current study showed the frequency of GG/AA genotype/phenotype in patients with sporadic CRC was more than in control group(42 vs. 19%)(OR = 3.08, CI_{95%} (1.27-7.44). Also this study suggests that no statistical difference(P>0.05) between GC/AP and CC/PP genotypes/phenotypes in patients group compared to controls. The results showed that the frequency of Arg allele was (0.66)(0.34) in patients and control group respectively, and found significant difference between Arg and Pro alleles in patients and controls(OR= 3.76), CI_{95%} (2.09-6.76). The main function of p53 protein is inducing of apoptosis in response to toxic environmental stimuli and this function is not complete when amino acid (Arg) substituted instead of amino acid (Pro) at the region rich Pro in structure of P53 protein^[23]. The Arg72 and Pro72 is not differs in the ability to binding with DNA but they have some biochemical and biological differences such as differs in their transcription activation mechanism and binding with different components of transcription factors^[24]. The present study disagreement with prior studies that failed to demonstrate the relationship between Arg72Pro polymorphism of TP53 gene and colorectal cancer^[25,26]. The Arg(G) allele was found to be increased genetic risk factor for colorectal cancer and this in agreement with the results of other studies^[27-30]. In the cell, P53 protein bind with DNA and stimulate another gene to synthesis of P21 protein that interact with a cyclin-dependent kinase inhibitor (cdk2)(cell division stimulating protein) and formation P21-cdk2 complex and here, the cell cannot pass through to the next stage of cell division and induce cell cycle arrest, normal DNA repair, differentiation, and apoptosis in response to oncogenic cellular stress such as carcinogen inducing DNA damage. If TP53 is mutate (e.g. Arg72Pro polymorphism), P21 protein is not available to act ((switch off)) for

cell division and growth suppression and the cells divided uncontrollably to form of the tumors^[31]. Table(4) shown significant statistical difference(decreasing) in TAO-C concentration of GG/AA genotype/phenotype in early stages of patients with sporadic CRC compared to advanced stages($p<0.05$). Many studies were showed that ROS are a known to cause oxidative nucleobase modifications in DNA (i.e., oxidized thymines, oxidized cytosines, oxidized adenines, oxidized guanines), which may lead to carcinogenesis^[32]. P53 protein involve in many pathways in body that increase production of ROS and P53 function as a transcription factors and this may be leads to DNA damage^[33]. The presence of GG genotyping may be induced decreasing the synthesis of an enzymatic antioxidants in advanced stages of disease. The findings of the present study indicate that TP53 codon 72 polymorphism may be a genetic predisposing factor for sporadic colorectal cancer and TP53 Arg72Arg(GG/AA genotype/phenotype) polymorphism was correlated with possible increased risk of this type of cancers in Babylon population^[34-36].

Conflicts of interest

The authors have no conflicts of interest to declare in relation to this article.

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