Molecular Evaluating the Therapeutic Application of Anticancer L-glutaminase Enzyme on Genetic Mutation Induced Rats

ABSTRACT

The Genomic DNA had been extracted from the liver tissue of the experimental rat groups following 15 weeks of treatment with partial purified L-glutaminase enzyme from E.coli in relation with ethyamine. The DNA samples of all the six treatment groups were amplified by PCR using three different primers specific for determining the presence of P53, bax and G3pdh genes and detect the effect of Ethylenimine and L-glutaminase on the animals at the molecular level. The agarose gel electrophoresis technique used to analyze the PCR amplification products. The result revealed the presence of P53 gene in all treatment groups except for diseased group (T2). This finding demonstrates the mutagenic effect of Ethylenimine that lead to mutation at P53 gene sequence, and therapeutic beneficial of L-glutaminase. Also, there is no PCR amplification product which represent Bax gene sequence for T2 and T5 groups which were administered doses of Ethylenimine, indicating that low doses of L-glutaminase failed to prevent the mutagenic effect of Ethylenimine. While, the G3pd genes is presented only in T2 and T5 groups which were administered doses of Ethylenimine. Finally, analysis the DNA sequences of the PCR amplified products extracted from liver samples of T2 group treated with Ethylenimine was carried out, then the results were compared with NCBI. The expected mutations were found at thirteen locals and there were only three mutated sequences with the DNA of liver samples from T5 group, while the DNA of animals in group (T6) were showed no mutated regions. The results of this novel study make clear the therapeutic effect of L-glutaminase and how suppress the mutagenic and carcinogenic effect of ethylenimine on P53, Bax, and G3pd genes, and its effect was dose dependent.

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KEY WORDS: L-glutaminase, Anticancer, ethylenimine, E.coli, PCR, DNA.

INTRODUCTION

Cancer is considered the most challenging disease while the main characteristic and challenging factor in most types of cancer is the inability of the cells to control their proliferation and loosing their normal regulatory mechanism that related to their division(Lai et al, 2020). There are many factors that playing role preventing cancer, and nutrition is considered one of the main factors which the last has been investigated by scientists and that has been leading recently to be considered of the most new approaches to protect and treating cancer (Wiseman, 2018). For instance, the tumor competes for the nitrogen compounds, and glutamine is considered such an effective transporter of nitrogen and carbon across different tissues among creatures (Yoo et al, 2020).

Moreover, glutamine metabolism in tumor cells has been found to be considerably faster than non-transformed cells of the same origin which that has been found in the recent studies on hepatocytes and hepatoma cells (Jiang et al, 2019). However, low level of glutamine might occur in some tumor...
regions that are distant from the source of blood supply which might explain the contribution of the low levels of glutamine to the cell death and necrosis in tumor (Knott et al, 2018).

Cancer cells that related to Acute lymphoblastic leukemia (ALL) cells is considered as an example for loosing cancer cells the ability to synthesize L-glutamine (Goto et al, 2014). As a result, the leukemic cells do not demonstrate the L-glutamine synthetase, thus, it depends on the exogenous source of L-glutamine which is important for their growth (DeBerardinis et al, 2007), as well as blood L-glutamine which serves as a metabolic precursor for the nucleotide and protein synthesis of tumor cells, so that, L-glutaminase (L-glutamine amidohydrolase EC 3.5.1.2) causes selective death of L-glutamine dependent tumor cells by blocking the energy route for their proliferation. (Sarada, 2013). Since L-glutaminase catalysis the hydrolysis of L-glutamine to glutamic acid and ammonia (Nandakumar, et al, 2003), therefore, can acts as a possible candidate for enzyme therapy (Goto et al, 2014).

The advantage of microbial glutaminase as their stability, large scale production over other sources, their role in biotechnological industries and their medical application as anticancer agent made microorganisms represent a preferable source for producing the enzyme and lead to continuous searching of high potential microorganisms strains (Habeeb, 2013). The aim of this study is the application of PCR, for detection the genetic mutation of the treated rat animal with Ethylenimine as mutagenic and carcinogenic agent, to improve the therapeutic beneficial of L-glutaminase against mutagenic effect.

MATERIALS AND METHODS

The extraction of total DNA from laboratory liver rats’ specimens, was performed according to the Qiagen DNeasy Blood & Tissue Kit procedure (Huang et al., 2009).

The estimation of both quality and quantity of extracted DNA

1- Agarose gel electrophoresis as an indicator for Estimating the quality of DNA

The quality of the DNA was assessed by analyzing through agarose gel electrophoresis. Agarose gels (1%) was made by adding 1gm of agarose to 100 ml of 1X TBE (tris boric acid e) buffer which solubilized by boiling temperature, then the agarose gel was left to cool down at 55˚C.

Later, Agarose gel poured in a plastic plate to solidify then toward the one of the ends for the gel , a comb was placed, and gel was left to be solidified. The gel plate was placed horizontally in electrophoresis tank where the buffer, 1XTBE was poured into gel tank, and 3µl of loading buffer was mixed with 10 µl DNA samples, which then followed by adding the samples carefully to the individual wells. Power supply was turned on at 5V/cm for 1-2 hours to run DNA. Ethidium bromide was used to stain the agarose gel by immersing in distilled water containing the dye for 30-45 minute with final concentration of 0.05µg/ml. DNA bands were visualized by U.V. transillumination at 366nm wave lengths on U.V. transilluminator, and gels were distained in distilled water for 30-60 minute to eliminate of the back ground staining. Then, photographing was done using a digital camera (Maniatis et al, 1982).

2-Estimating the quantity and purity of DNA by UV Spectrophotometer

Use 1µL of the 1:20 dilution to estimate the DNA concentration spectrophotometrically. A volume of 20 µl of extracted DNA was added to 1980 µl of TE buffer and mixed thoroughly then optical density (OD) was measured using a spectrophotometer at wavelengths of 260 to 280 nm. The DNA concentration was calculated according to the following formula :

\[
\text{DNA concentration (µg /µl)} = \frac{\left[\text{OD260} \times \text{dilution factor} \times 50 \, \mu\text{g/ml}\right]}{1000} = \frac{\left[\text{OD260} \times 100 \times 50 \, \mu\text{g/ml}\right]}{1000}
\]

Theoretically, OD260 of one corresponds to approximately (50µg/ml) for double-strand DNA. The ratio between the reading at 260nm /280nm provides estimate of the purity of nucleic acid. DNA is considered pure and acceptable for use with the OD between 1.8 to 2.0 which is a good indicator to measure the purity of nucleic acid (Sambrook et al, 1989).

Polymerase Chain Reaction (PCR):

Since the high sensitivity of the PCR, an extra adequate measures has been taken to avoid contamination with other DNA sources which may present in the lab. The DNA sample preparation
reaction mixture was assembled. PCR process, as well as the subsequent product analysis are performed in separated areas. In order to prepare the reaction mixture, a laminar flow cabinet provided with UV lamp was used while fresh gloves with micropipettes and sterile tips were provided for each step of PCR reaction. The reagent for PCR is prepared separately with ice and used solely for this purpose and optimum concentration was used. Aliquots are stored separately from other DNA samples (Mulhardt, 2007).

1. Preparation of primers:

PCR reactions were performed using the following specific primers. Three oligonucleotide primers sequence were used, synthesized by Thermo Fisher Scientific in a lyophilized form. These primers were dissolved in a sterile deionized distilled water to give a final concentration of 10pmol/µl as recommended by provider. The name of primers with sequences are listed in Table (1).

| Gene name                         | Primer Name | Sequence (5' - 3')                          |
|-----------------------------------|-------------|--------------------------------------------|
| The tumor suppressor gene p53    | p53         | F 5’. AAG ACA TGC CCT GTGCAG TT.3’        |
|                                   |             | R 5’. GAG TCT TCC AGC GTG ATG AT.3’       |
| Apoptosis regulator BAX gene     | Bax         | F 5’. CCG AGA GGT CTT CCG TGT G.3’        |
|                                   |             | R 5’. GCC TCA GCC CAT CTT CCA.3’          |
| Glyceraldehyde.3 P-Dehydrogenase  | g3pdh       | F 5’. ATG GTGAAG GTC GGTGTG AACG.3’       |
|                                   |             | R 5’. GTT GTC ATG GAT GACCTT GGC C.3’     |

2-PCR Master Mix:

PCR Master Mix was thawed at room temperature, then mixed gently with vortex apparatus, and finally spin down shorty in a micro centrifuge to collect the material at bottom of the tube. While master mix is a ready mixture for use which, contains TaqDNA polymerase, MgCl2, pure deoxy nucleosides (dNTPs), reaction buffer that increases sample density. In order to achieve homogeneity of reagents and reduce the risk of contamination, amplification was performed under aseptic conditions inside the hood. The master mix was prepared containing for all samples and it contain all the components of the reaction except of template DNA which was mixed gently with deionized distilled water to achieve the appropriate volume. Genomic DNA Amplification of genomic DNA was performed with the following master amplification reaction:

| No.  | Component             | Final Concentration | Volume  |
|------|-----------------------|---------------------|---------|
| 1    | PCR master mix        | 1X                  | 5 µl    |
| 2    | DNA template sample   | 50ng                | 1 µl    |
| 3    | Forward primer        | 10pmol/l            | 1 µl    |
| 4    | Reverse primer        | 10pmol/l            | 1 µl    |
| 5    | Sterile distilled water | -                 | 17µl   |
|      | Final reaction volume |                     | 25µl    |
The components mentioned in table 2, are mixed well by vortex, then all samples were transferred into the thermal cycler. Ultimately, the PCR program is started as mentioned in table 3, for each primer.

3-Agarose gel electrophoresis for PCR products

10µl of PCR amplified products and 3µl of loading dye were separated by electrophoresis in 1% agarose gels (The electrophoresis was carried out for 90 minutes, while 60 mA, 45 volt for small tank and 90 minutes, 90 mA, and 60 volt for the large tank, for products' separation). Gels were stained with ethidium bromide; UV transilluminator was used to visualize PCR products and digital camera was used for imaging. DNA ladder (100)bp was used as a source to compare to the amplified products. (Sambrook et al, 1989).

RESULTS AND DISCUSSION

Extraction of Genomic DNA

The Genomic DNA had been extracted from the liver tissue of the experimental rat groups following 15 weeks of treatment with different concentration from Ethylenimine (mutagenic agent) in relation with partial purified L-glutaminase enzyme obtained from E.coli in our previous research (Karim et al, 2016). The concentration of isolated DNA samples from all the treatment groups ranged from 351.5-649.2 ng/µl (Table 4). Their purity ranged from 1.85-1.94 estimated by UV spectrophotometer with high molecular size, determined by using 1% agarose gel electrophoresis. These results indicated the valuable yield of DNA that is suitable for PCR amplification and revealed the advantage of the method used in the study. This finding might be due to the chemicals included in the kit from Qiagen company. For instance, the used Proteinase K which is one compound of the kit used for protein denaturation leaving the nucleic acid in the aqueous phase to be ethanol precipitated, in addition to the exact application of the procedure and using optimum concentration of reacted material and performing sterilization technique.

Table (3) PCR amplification program

| Steps | NO. of cycles | Characters       | Temp./°C | Time   |
|-------|--------------|-----------------|----------|--------|
| Step 1 | 1 cycle      | Initial denaturation | 94°C     | 4min.  |
| Step 2 | 35 cycles    | Denaturation steps | 92°C     | 0.5min.|
|        |              | Annealing steps  | 36.5°C   | 0.5min.|
|        |              | Extension steps  | 72°C     | 2min.  |
| Step 3 | 1 cycle      | Final Extension  | 72°C     | 7min   |
| Step 4 | Hold         |                 | 4°C      | ----   |

The thermal cycler was held at 4°C.
Table (4) Purity of DNA from liver tissue of rat's subcutaneous injection by L-glutaminase and orally of Ethylenimine for 15 weeks

| Treatment Types | DNA Conc. ng/μl | Purity |
|-----------------|------------------|--------|
| T1              | 745.4            | 1.88   |
| T2              | 649.2            | 1.91   |
| T3              | 351.5            | 1.89   |
| T4              | 538.3            | 1.94   |
| T5              | 463.7            | 1.85   |
| T6              | 528.3            | 1.91   |

Application of Polymerase Chain Reaction (PCR) for detection of the P53, Bax and G3pdh genes

The PCR reaction conditions optimized, the reliable concentration of DNA for PCR amplification was estimated by using serial dilution of purified DNA samples with Distilled water and 1µl of each dilution used in PCR technique. The DNA samples of all the six treatment groups were tested using three different primers (Table 1) specific for determining the presence of P53, bax and G3pdh genes respectively, and detect the interaction effect of Ethylenimine and L-glutaminase on the animals at the molecular level. To improve the successful application, the agarose gel electrophoresis technique used to analyze the PCR amplification products. The Ethidium bromide-stained gel then was visualized by UV-transilluminater and photographed.

As shown in Fig. (1), agarose gel electrophoresis revealed the presence of a single band with molecular size of 367 bp. At the lane number; 1, 3, 4, 5, 6 which represent the treatment (T); T1, T3, T4, T5, and T6 respectively. Whereas there is no band seen at the lane 2, which represent the T2 (the diseased group which administered 1mg Ethylenimine / Kg. rats' body weight. This finding demonstrates the mutagenic effect of Ethylenimine that lead to mutation at P53 gene sequence, so that the P53 primers failed to amplify the mutated sequences. Hence there are no PCR amplification products. However, The T5 and, T6 groups are also administered 1mg Ethylenimine / Kg. rats' body weight but in combination with 0.5ml and 1ml of L-glutaminase (with 70 IU/ml enzymatic activity) respectively. This result makes clear the therapeutic effect of L-glutaminase and how it was suppressed the mutagenic activity of ethylenimine on P53 gene.

Fig. (1) DNA profiles in 1% agarose gel electrophoresis of PCR products, oligonucleotides primer was used for detection of P53 gene in experimental animals

T1: control group; T2: 0.5mg Ethylenimine/kg; rats' body weight; T3: Glutaminase 0.5ml/kg; T4: Glutaminase 1ml/kg; T5: 0.5ml/kg Glutaminase+0.5mg Ethylenimine/kg; T6: 1ml/kg Glutaminase+0.5mg ethylenimine/kg.
As shown in agarose gel electrophoresis Fig.(2), there is a single band with a molecular size of 318bp. at the lane number; 1, 3, 4, and 6 which represents the T1, T3, T4 and T6 respectively. Whereas, there are no DNA bands at lane 2 and 5 which represent T2 and T5 respectively. This observation revealed the mutation at the Bax gene sequence attributed to administrating doses of ethylenimine by T2 and T5.

Although T6 also administered doses of Ethylenimine but the band of amplified Bax gene sequence have been produced, since the dose of L-glutaminase enzyme has been duplicated for T6 in contrast to that administered by T5. This observation reflects the role of L-glutaminase in protecting the Bax gene from mutagenic activity of ethylenimine and it was dose dependent.

![Agarose gel electrophoresis Fig. (2)](image2)

Fig. (2) DNA profiles in 1% agarose gel electrophoresis of PCR products, oligonucleotide primer was used for detection of Bax gene in experimental animals.

T1: control group; T2: 0.5mg Ethylenimine/kg; T3: Glutaminase 0.5ml/kg; T4: Glutaminase 1ml/kg; T5: 0.5ml/kg Glutaminase+0.5mg Ethylenimine/kg; T6: 1ml/kg Glutaminase+0.5mg ethylenimine/kg.

Agarose gel electrophoresis Fig. (3) shows the appearance of a single band with a molecular size of 495bp. at the lane 2 and 5, which represent the T2 and T5 respectively. However, there are no DNA bands at the other lanes. This finding might be attributed to the activation of the G3pdh gene as a result of administrating oral doses of ethylenimine. Since this gene became highly expressed in liver cells affected by toxigenic or mutagenic agents and was specific indicator of tumor developed in the liver cells.

![Agarose gel electrophoresis Fig. (3)](image3)

Fig. (3) DNA profiles in 1% agarose gel electrophoresis of PCR products, oligonucleotide primer was used for detection of G3pdh gene in experimental animals.
T1: control group; (T2: 0.5mg Ethylenimine/kg; T3: Glutaminase 0.5ml/kg; T4: Glutaminase 1ml/kg; T5: 0.5ml/kg Glutaminase+0.5mg Ethylenimine/kg; T6: 1ml/kg Glutaminase+0.5mg ethylenimine/kg.)/rats body weight.

P53 defective could lead to cancer by allowing defective cells to divide without control, which resulting in cancer and as has been indicated in recent studies that around 50% of all human tumors contain p53 mutants. However, in liver cancer, Tp53 which thought to be eliminated along with some other genes at early stage of tumorigenesis (Lacova, et al, 2011), so that P53 DNA biomarker suggested for the early detection of tumors with mutated TP53 which the last has been detected in sera and other body fluids. For instance, the patients with liver cancer, their DNA containing mutations in TP53 gene which the last can be found in their serum (Kirk et al, 2005).

Genotoxicity of ethylenimine had been investigated, and an increased in the frequencies of chromatid breaks/gaps has been observed in fibroblast cells which the last incubated with $10^{-5}$ M ethylenimine (Chang and Elequin, 1967). Ethylenimine was tested to detect gene toxicity in about 150 species by Ramel (1981), who concluded that it is a very potent direct-acting mutagen, producing point mutations and chromosome aberrations. In 1999, the potential carcinogenicity of ethylenimine was evaluated by International Agency for cancer Research and concluded that ethylenimine is possibly carcinogenic to humans (Group 2B). Since ethylenimine is a direct-acting alkylating agent which as a result is considered as mutagenic in different living organism such as in vitro cell culture, bacteria, insects, and other animals (in vivo).

These findings improved the preventive role of the L-glutaminase against the mutagenic effect of ethylenimine, and it was dose dependent. Pandian et al, (2014) reported that purified L-glutaminase from Alkaligene faecalis KLU102 exhibit a dose-dependent cytotoxic activity against HeLa cells, with an IC50 value of 12.5µg/ml. The role of L-glutaminase against cancer, represented by reducing cell viability which contributes mainly to protect, prevent and arresting the progressing of cancer. The mutation types for the P53 gene included the deletion, frame shift and insertion mutations. While the Bax gene contained only frameshift mutation and the G3pdh gene contained insertion mutation. The results of this novel study make clear the therapeutic effect of L-glutaminase and how it suppresses the mutagenic and carcinogenic effect of ethylenimine on P53, Bax and G3pd genes. The mutation in TP53 gene causes loss of function that performed by this gene which includes; acting as a tumour suppressor gene, participates in many cellular functions: cell cycle control, DNA damage and repair, gene transcription and cell apoptosis. Moreover, many P53 mutants are able to actively promote tumour development by several other means (Milner et al, 1991).

It has been found that glutaminase purified from a gram positive coccus and other sample from gram negative rods with considerable lower km value resulted in marked inhibition of Ehrlich ascites carcinoma when given 24 hours after tumour implantation and increase the survival time of tumour bearing animals (Robert et al, 1970).

Nathiya et al, (2012) reported the cytotoxicity of L-glutaminase obtained from Aspargillus flavus KUGFOO9 to words breast cancer cell lines (IC50 250µg/ml). Singh and Banik, (2013) were reported antitumor activity of L-glutaminase produced by Bacillus cereus MTCC1305.They observed the gradual inhibition in growth of hepatocellular carcinoma (Hep-G2) cell lines was found with IC50 value of 82.27 g/ml in the presence of different doses of L-glutaminase enzyme. However, the purified intracellular L-glutaminase from Penicillum brevicompactum NRC829 inhibited the growth of human cell line hepatocellular carcinoma (Hep-G2) with IC50 value of 63.3µg/ml (Elshafei et al, 2014).

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المضاد للإنسان على الفئران الناجمة عن الطفرة الوراثية L-glutaminase

الخلاصة

تم استخراج الحمض النووي الجينومي من أنسجة الكبد لمجموعات الفئران التجارية بعد 15 أسبوعًا من التجربة. تم استخدام PCR باستخدام تسلسلات ثلاثية G3pdh و Bax و P53 لمعرفة تأثير الإيثيلينيمين Ethylenimine على النتائج. استخدمت تقنية PCR لتحليل الجينات والإنزيمات. تم تحليل النتائج باستخدام ألكاروز agarose، وتم فحص النتائج أيضًا باستخدام الدراسات المختبرية لـ L-glutaminase. نتيجة هذه الدراسات، لا يوجد تأثير لـ Ethylenimine على النتائج. النتائج تشير إلى أن الجينات المضادة للمضادات للسرطان، مثلاً P53 و Bax، لا تتأثر بـ Ethylenimine. يمكن أن تساعد هذه الدراسات في فحص تأثيرات الإيثيلينيمين على النزاعات الخلوية والجينومية.