Tikrit Journal for Agricultural Sciences (2021) 21 (4):159-167
https://doi.org/10.25130/tjas.21.4.17

IRAQI Academic Scientific Journals
العراقية المجلات الأكاديمية العلمية

ISSN:1813-1646 (Print); 2664-0597 (Online)
Tikrit Journal for Agricultural Sciences
Journal Homepage: http://www.tjas.org
E-mail: tjas@tu.edu.iq

TJAS
Tikrit Journal for Agricultural Sciences

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KEY WORDS:

L-glutaminase, Anticancer, ethylenimine, E.coli, PCR, DNA.

ARTICLE HISTORY:

Received: 15/12/2020
Accepted: 31/12/2020
Available online: 31/12/2021

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 ethylamine. 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. 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..

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 trprotect 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

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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. Ethidum 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 :

DNA concentration (µg /µl) = [OD260*dilution factor*50 µg/ml] /1000

= [OD260*100*50 µg/ml] /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 assembeled ,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).

Table (1) Names of the primers with their sequences which used in the study

Gene name	Primer Name	Sequence (5́ - 3′)
The tumor suppressor gene	*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:

Table (2) PCR master mix materials

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**

Table (3) PCR amplification program

Steps	NO. of cycles	Characters	Temp./°C	Time
Step 1	1 cycle	Initial denaturation	94°C	4min.
Step 2	35cycles	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 components mentioned in table 2, are mixed well by vortex, then all samples were transfered 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 visulize PCR products and digital camera was used for imaging. DNA ladder (100)bp was used as asource 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 (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 0.5m1 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.

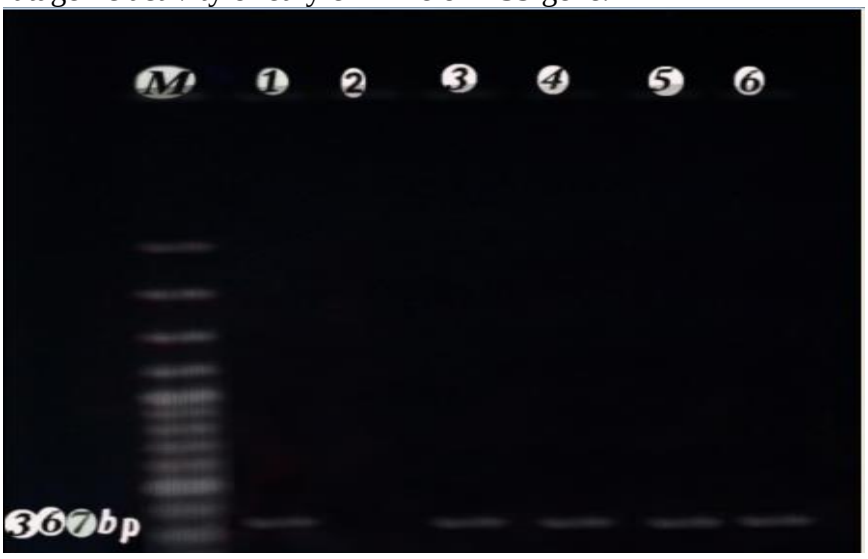

Fig. (1) DNA profiles in 1% agarose gel electrophoresis of PCR products, oligonucleotides primer was used for detection of *P53* gene in experimental animals

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.

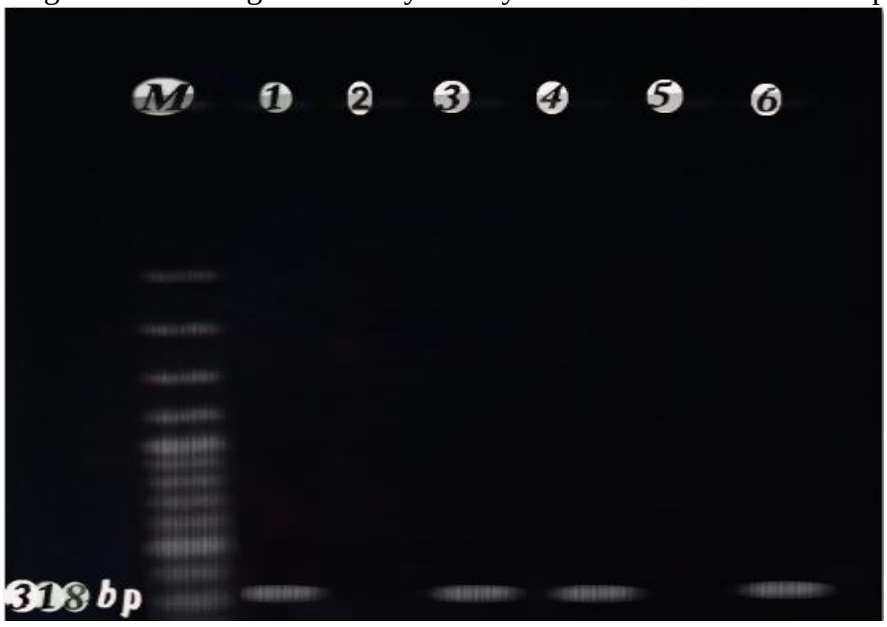

Fig. (2) DNA profiles in 1% agarose gel electrophoresis of PCR products, oligonucleotide primer was used for for detection of Bax gene in experimental animals.
T1: control group; T2: 0.5mg Ethylenimine/kg; T3: Glutaminase 0.5ml/kg; T4: Glutaminase 1ml/kg; T5: 0.5ml/kg Glutaminase+0.5mg Ethylenimine/kg; 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.

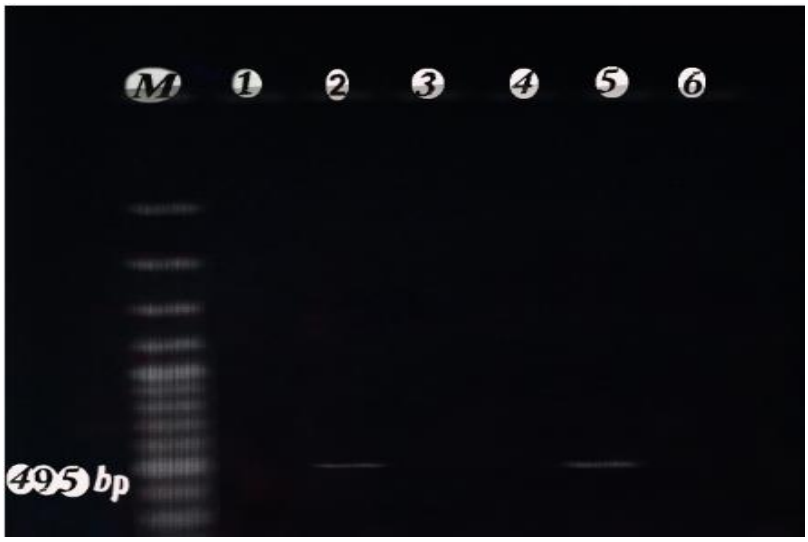

Fig. (3) DNA profiles in 1% agarose gel electrophoresis of PCR products, oligonucleotide primer was for detection of *G3pdh* gene in experimental animals.

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 ethylinimine, 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 Ehrilich 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 *Penicillium 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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التقييم الجزيئي للتطبيق العلاجي لأنزيم L-glutaminase المضاد للانسان على الفئران الناجمة عن الطفرة الوراثية

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الخلاصة

تم استخراج الحمض النووي الجينومي من أنسجة الكبد لمجموعات الفئران التجريبية بعد 15 أسبوعا من العلاج مع إنزيم L-glutaminase المنقى جزئيا من بكتريا القولون فيما يتعلق بالأثيل امين. تم تضخيم عينات الحمض النووي لجميع مجموعات المعاملات الست بواسطة PCR باستخدام ثلاثة مبرمات مختلفة محددة لتحديد وجود جينات P53 و Bax وG3pdh والكشف عن تأثير الإيثيلينيمين وL-glutaminase على الحيوانات على المستوى الجزيئي. استخدمت تقنية هلام الإلكتروفورسيس agarose لتحليل منتجات تضخيم PCR، وكشفت النتيجة عن وجود جين P53 في جميع مجموعات العلاج باستثناء المجموعة المريضة (T2). يوضح هذا الاكتشاف التأثير المطفر للإيثيلينيمين الذي يؤدي إلى حدوث طفرة في تسلسل الجين P53 ، والفائدة العلاجية لـ L-glutaminase. أيضًا ، لا يوجد منتج تضخيم PCR والذي يمثل تسلسل جين Bax لمجموعات T2 و T5 التي تم إعطاؤها جرعات من Ethylenimine ، مما يشير إلى أن الجرعات المنخفضة من L-glutaminase فشلت في منع التأثير المطفر للإيثيلينيمين. بينما وجدت جينات G3pd فقط في T2 و T5. أخيرًا ، تم إجراء تحليل تسلسل الحمض النووي للمنتجات المتضخمة PCR المستخرجة من عينات الكبد من مجموعة T2 المعالجة بإيثيلينيمين ، ثم تمت مقارنة النتائج مع NCBI. تم العثور على الطفرات المتوقعة في ثلاثة عشر من السكان المحليين ولم يكن هناك سوى ثلاثة متواليات متحولة مع الحمض النووي لعينات الكبد من مجموعة T5 ، بينما لم يظهر الحمض النووي للحيوانات في المجموعة (T6) أي مناطق متحولة. توضح نتائج هذه الدراسة الجديدة التأثير العلاجي لـ L-glutaminase وكيف قمع التأثير المطفر والمسرطن لإيثيلينيمين على جينات P53 و Bax و G3pd ، وكان تأثيره يعتمد على الجرعة.

الكلمات المفتاحية: L-الجلوتاميناز ، مضاد للسرطان ، إيثيلينيمين ، بكتريا القولون ، تفاعل البوليميرز المتسلسل ، الحمض النووي.