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The Evaluation for Anticancer Activity on Hep-2 and HepG-2 Cells of Ribosomal Protein L36 (RPL36) from the *Escherichia coli* BL21

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Cancer is one of the leading causes of mortality worldwide. Many of the protein from bacterium possess antitumor activity. The present study was carried out to evaluate the anti-cancer activity of Ribosomal Protein L36 (RPL36) from the *Escherichia coli* BL21on human laryngeal carcinoma Hep-2 cells and Human hepatoma Hep G-2 cells. Evaluation of the effect of RPL36 response was made by the study of tumor growth response including cell morphology. The human laryngeal carcinoma Hep-2 cells treated with 0.3125-10μg/ml of RPL36 for 24 hours displayed significant cell growth inhibition ($p < 0.05$, $n = 6$) in assayed using MTT compared to the control (untreated) cells. For comparison, human hepatoma Hep G-2 cells displayed no significant change ($p > 0.05$; $n = 6$) when compared to the control (untreated) cells. The RPL36 has a time and dose dependent on Hep-2 cells growth inhibition. The data indicate that the effect at low concentrations is better than high concentrations, and the concentration of 0.625¼g/ml has the best rate of growth inhibition. The Real Time PCR array analysis of gene expression profile indicated that the anti-cancer activities of RPL36 on Hep-2 cells may involve DAPK1, DAPK3 and FADD signaling pathway for cell Apoptosis. The results of this study support the efficacy of *Escherichia coli* BL21ribosomal protein L36 as an anticancer agent for human laryngeal carcinoma Hep-2 cells. However, further studies of the mechanism and the signal transduction pathways are required.

Key words: Anticancer activity, RPL36, Escherichia coli BL21, Hep-2 cells, Hep G-2 cells.

Escherichia coli commonly abbreviated *E. coli*) is a Gram-negative, rod-shaped bacterium that is commonly found in the lower intestine of warm-blooded organisms (endotherms). The genus belongs in a group of bacteria informally known as "coliforms", and is a member of the Enterobacteriaceae family ("the enterics") of the Gammaproteobacteria[1]. *E. coli* and related bacteria constitute about 0.1% of gut flora, and fecal-oral transmission is the major route through which pathogenic strains of the bacterium cause disease[2, 3]. The bacterium can also be grown easily and inexpensively in a laboratory setting, and has been intensively investigated for over 60 years[4] *E. coli* is the most widely studied prokaryotic model organism, and an important species in the fields of biotechnology and microbiology, where it has served as the host organism for the majority of work with recombinant DNA[5,6]. Most *E. coli* strains are harmless, but some serotypes can cause serious food poisoning in humans, and are occasionally responsible for product recalls. The harmless strains are part of the normal flora of the gut, and can benefit their hosts by producing vitamin K2, and by preventing the establishment of pathogenic

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bacteria within the intestine[1]. Nonpathogenic Escherichia coli BL21strain Nissle 1917 also known as Mutaflor is used as a probiotic agent in medicine, mainly for the treatment of various gastroenterological diseases, including inflammatory bowel disease [7]

The ribosome of *E. coli* has about 22 proteins in the small subunit (labelled S1 to S22) and 34 proteins in the large subunit (L1 to L36)[9]. Except for S1 (with a molecular weight of 61.2 kDa), the other proteins range in weight between 4.4 and 29.7 kDa[10]. Ribosomal protein L36 of Escherichia coli BL21located in the large ribosomal 50S subunit, encoding by the rpmj gene with the other names, such as B3299, SecX, RpmJ *et al* [10].

Cancer, known medically as a malignant neoplasm, is a term for a large group of different diseases, all involving unregulated cell growth [11]. In cancer, cells divide and grow uncontrollably, forming malignant tumors, and invade nearby parts of the body. The cancer may also spread to more distant parts of the body through the lymphatic system or bloodstream[11]. Cancer is one of the most fatal diseases in human population and one of the most frequent causes of death worldwide[12, 13]. Many of the protein from bacterium possess antitumor activity. The present study was carried out to evaluate the anti-cancer activity of Ribosomal Protein L36 (RPL36) from the *Escherichia coli* BL21BL21.

MATERIALS AND METHODS

Materials and DNA isolation

Total DNAs were isolated from the Escherichia coli BL21using the Total Tissue/Cell RNA Extraction Kits(Waton Inc., Shanghai, China) according to the manufacturer's instructions. The total DNAs extracted were dissolved in diethypyrocarbonate (DEPC) water and kept at -70ºC.

Primers design, RT-PCR, cloning of RT-PCR products and Sequencing

The PCR primers were designed by Primer Premier 5.0, basing on the DNA sequence of *RPL36* from Escherichia coli BL21K-12 substr. The specific primers of DNA sequence are as follows:

RPL36-F: 5' -ATGAAAGTTC GTGCTTCC-3'

RPL36-R: 5' -TCAGCCTTGG CGCTGTTTAT -3'

Total DNAs were synthesized into the first-stranded DNAs using a reverse tran-scription kit with Oligo dT as the primers, according to manufacturer instructions (Pro-mega). A 50µl volume of first-strand DNA synthesis reaction system included 5µl total DNA, 3µl MgCl2 (2.5mM), 4µl dNTPs (2.5 m), 1µl of each primer, 0.25µl Taq Plus DNA polymerase (Sangon Co., Shanghai, China) (5U/µl). A amplification was performed using an MJ Research thermocycler, Model PTC-200 (Watertown, MA, USA) with a program of 4 min at 94.0°C, followed by 30 cycles of 1 min at 94.0°C, 0.5 min at 45°C and 1.5 min at 72.0°C, and then ended with a final extension for 10 min at 72.0°C. After amplification, PCR products were separated by electrophoresis on a 1.5% agarose gel with 1X TAE (Tris-acetate-EDTA) buffer, stained with ethidium bromide and visualized under UV light. The expected fragments of PCR products were harvested and purified from the gel using a DNA harvesting kit (Omega, China), and then ligated into a pET28a vector at 22°C for 12 h. The recombinant molecules were transformed into Escherichia coli BL21complete cells (JM109), and then spread on an LB-plate containing 50 µg/mL ampicillin, 200 mg/mL IPTG (isopropyl-beta-D-thioga-lactopyranoside), and 20 mg/mL X-gal. Plasmid DNA was isolated and digested by *Pst*I and *Sca*II to verify the insert size. Plasmid DNA was sequenced by Huada Zhongsheng Scientific Corporation (Beijing, China).

Chemical synthesis of RPL36 protein

The RPL36 protein was synthesized use chemical method by Shanghai Shenggong Scientific Corporation (Shanghai, China)

Cell lines

Human laryngeal carcinoma Hep-2 cells and human hepatoma Hep G-2 cells were purchased from North Sichuan Medical College, Institute of Biochemistry and Molecular Immunology and maintained for study in MEM (Gibco, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS, Evergreen biological Products company, China)0100U/ml penicillin (Gibco Company USA) 0100µg/ml streptomycin (Gibco Company USA) 0pH7.4,,vRPMI-1640(Gibco Company USA) Cells were cultured in a 5% CO_2/95% air incubator at 37ºC.

MTT Assay for cell viability

The MTT reagent is ready for use. To prevent contamination, aseptically remove the

appropriate volume for use during the entire experiment and place it into a separate tube. Return the remainder to storage at 2 - 8° C in the dark. Contamination will compromise the stability of this reagent. Suspension cells were harvested by centrifugation at 500 × g for 5 minutes at 2 - 8° C. Adherent cells should be released from their substrate by trypsinization or scraping. Pellet cells by centrifugation at 500 × g for 5 minutes at 2-8° C. Discard supernate.Wash cells by resuspending in 5 mL sterile PBS. Pellet cells by centrifugation at 500 ´ g for 5 minutes at 2-8° C. Discard supernate. Resuspend cells at 5 x 10^6 cells/mL in tissue culture medium. Harvest and wash sufficient cells to prepare 8-10 serial two-fold dilutions with 100 L of cells/well, in triplicate. Serially dilute cells using 5 mL culture tubes. Plate cells at 100 L/well. Then add the ribosomal protein L36 of Escherichia coli BL21to a final concenteation of 0, 10, 5, 2.5, 1.25, 0.625, 0.5, 0.3125 µg/ml include 6 repeated holes and 3 control wells of cell culture medium alone. Incubate the cells for 24 hours. Add 10 L of MTT reagent to each well. Transfer the full amount required for the entire experiment to a separate tube and return the stock to storage at 2-8° C in the dark. Incubate the plate for 2 - 4 hours at 37° C. View the cells periodically for the appearance of punctate, intracellular precipitate using an inverted microscope.

When purple precipitate is clearly visible under the microscope, add 100 L of Detergent Reagent to all wells, including control wells. Do not shake. Leave covered plate in the dark at 18-24° C for at least 2 hours to overnight. Samples may be read after 2 hours, but if the readings are low and there are crystals remaining return the plate to the dark and incubate for a longer period. Remove the plate cover and measure the absorbance of the wells, including the Blanks at 490 nm. The blanks should give values of 0.1 O.D. units. The absorbance range for untreated cells should typically be between 0.75 and 1.25 O.D. units. Determine the average values from triplicate readings and subtract the average value for the blank. Placed the 96-well plates under inverted microscope, camera records different concentrations changes for cell morphology.

Cytokines determination

The amount of DAPK1, DAPK3 and FADD of culture supernatants were determined by ELISA kit (purchased from R&D Systems) following the instruction of manufacturers.

Quantitative RT-PCR detection of related gene expression

The peritoneal macrophages were harvested after stimulated by various concentration of RPL36 for 4 h. The total cellular RNA was extracted using Trizol Reagent (Invitrogen, USA) and reverse-transcribed into cDNA using oligo $(dT)_{18}$ primers (Invitrogen, USA). Amplification of each target cDNA was performed in the icycler system (Bio-rad, USA). PCR products were quantified using SYBR Green I. β-actin was used as an endogenous control to normalize expression levels among samples. A standard curve of each primer set was generated using LPS-induced macrophage cDNA. The PCR primers chosen were shown in Table 1. The relative expression abundance was calculated by the following formula:

Relative expression abundance= mols of detected mRNA/ mols of β-actin mRNA

Data analysis

The sequence data were analyzed by GenScan software (http://genes. mit.edu/GENSCAN.html). Homology research of the Ribosomal Protein L36 (RPL36) from the Escherichia coli BL21compared with the gene sequences of other species ware performed using Blast 2.1 (http://www.ncbi.nlm.nih.gov/blast/). ORF of the DNA sequence was searched using ORF finder software (http://www. ncbi.nlm.nih.gov/gorf/gorf.html). Secondary Structure Prediction of the RPL36 sequence was searched using APSSP2 software (http://www.imtech.res.in /raghava/apssp/). Tertiary structure Prediction of the RPL36 sequence was searched using SWISS-MODEL software (http://swissmodel.expasy.org/). Protein structure of the RPL36 sequence loned was deduced using PredictProtein software (http://cubic.bioc.columbia.edu/predictprotein/).

Statistical analysis

The data were expressed as means ± SD. The significance of difference was evaluated with one-way ANOVA, followed by Student's t-test to statistically identify differences between the control and treated groups. Significant differences were set at $P < 0.05$ and $P < 0.01$.

RESULTS AND DISCUSSION

Analysis of the cDNA of RPL36 from the Escherichia coli BL21

About 120 bp of DNA fragment was amplified from the Escherichia coli BL21. The length of the DNA cloned is 117 bp. Blast research showed that the DNA sequence cloned is highly homologous with the *RPL36* from Escherichia coli BL21K-12 substr.reported. On the basis of the high identity, we concluded that the DNA isolated is the DNA encoding the Escherichia coli BL21RPL36 protein. An ORF of 114bp encoding 38 amino acids was found in the DNA sequence (Figure 1).

Prediction and analysis of primary structure0protein functional sites and advanced structure in RPL36 protein

Primary structure analysis revealed that the molecular weight of the putative RPL36 protein of the Escherichia coli BL21is 4.364 kD with a theoretical pI 11.40. Secondary and tertiary Structure analysis indicated that there is 13.158% E (strand); 26.316% H (helix) and 60.526% C (coil) (Figure 2). Topology prediction shows that there is only one Ribosomal protein L36 signature site which located from 11 to 37 amino acid residues in the RPL36 protein of the Escherichia coli. The functional site is in the hydrophobic and hydrophilic regions which both with the greatest change (Figure 3). The protein sequence shows a homology to those of other strains. No matter what differences are there, they have the same functional site: Ribosomal protein L36 signature.

Anticancer activity of RPL36 on human laryngeal carcinoma Hep-2 cells and human hepatoma Hep G-2 cell

Laryngeal cancer may also be called cancer of the larynx or laryngeal carcinoma. Most laryngeal cancers are squamous cell carcinomas, reflecting their origin from the squamous cells which form the majority of the laryngeal epithelium. Laryngeal cancer may spread by direct extension to adjacent structures, by metastasis to regional cervical lymph nodes, or more distantly, through the blood stream. Distant metastates to the lung are most common [14]. STR (DNA)-profiling has revealed that the Hep-2 cell line is almost identical to the HeLa cell line[15,16]. A hardy cell line, Hep-2 resists temperature, nutritional, and environmental changes without a loss of viability. It has supported growth of 10 of 14 arboviruses and measles virus, and it has been used for experimental studies of tumor production in rats, hamsters, mice, embryonated eggs and volunteer terminal cancer patients [17, 18]. While Hep G2 (Hepatocellular carcinoma, human) cells are epithelial in morphology, which secrete a variety of major plasma proteins; e.g., albumin, transferrin and the acute phase proteins fibrinogen, alpha 2-macroglobulin, alpha 1-antitrypsin, transferrin and plasminogen[19]. Hepatitis Bvirus surface antigens have not been detected. The cells will respond to stimulation with human growth

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1  atgaaagttcgtgcttccgtcaagaaattatgccgtaactgcaaa
     M  K  V  R  A  S  V  K  K  L  C  R  N  C  K
46 atcgttaagcgtgatggtgtcatccgtgtgatttgcagtgccgag
     I  V  K  R  D  G  V  I  R  V  I  C  S  A  E
91 ccgaagcataaacagcgccaaggctga 117
     P  K  H  K  Q  R  Q  G  *
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Fig. 1. Nucleotide and deduced amino acid sequences of DNA enoding the *Escherichia coli* BL21 RPL36 protein

Fig. 2. Tertiary structure of RPL36 protein

hormone and express 3-hydroxy-3-methylglutaryl-CoA reductase and hepatic triglyceride lipase activities. They demonstrate decreased expression of apoA-I mRNA and increased expression of catalase mRNA in response to gramoxone (oxidative stress) and have the insulin and insulin-like growth factor II (IGF II) Receptors[20].

Our experiments get the different results because of the differences in these two kinds of cancer cells. As shown in Figure 4, the human laryngeal carcinoma Hep-2 cells treated with 0.3125-10μg/ml of RPL36 for 24 hours displayed significant cell growth inhibition ($p < 0.05$, $n = 6$)in assayed using MTT compared to the control (untreated) cells, while human hepatoma Hep G-2 cells displayed no significant change ($p > 0.05$; $n = 6$) (Fig. 5). The data indicate that the effect at low concentrations is better than high concentrations, and the concentration of 0.625μg/ml has the best rate of growth inhibition (Fig. 4) . Topology prediction shows that there is only one Ribosomal protein L36 signature site in the RPL36 protein of the *Escherichia coli* BL21BL21. The protien sequence shows a homology to those of other strains. No matter what differences are there, they have the same functional site: Ribosomal protein L36 signature. The functional site is in the hydrophobic and hydrophilic regions which both with the greatest change. In aqueous solution, the exposure of large hydrophobic surface regions is energetically unfavourable. Hydrophobic side chains tends to be buried in the middle of the protein, whereas hydrophilic side chains are exposed to the solvent. The hydrophobic part of the solvent-accessible surface of a typical monomeric globular protein consists of a single, large interconnected region formed from faces of apolar atoms and constituting-60% of the solvent-accessible surface area. Therefore, the direct delineation of the hydrophobic surface patches on an atom-wise basis is impossible. The remaining hydrophobic surface pieces that probably play an important role in intra- and inter- molecular recognition processes such as ligand binding, protein folding and protein–protein association in solution conditions.

The effect of RPL36 on morphology of human laryngeal carcinoma Hep-2 cells and human hepatoma Hep G-2 cell

The 96-well plates were placed under inverted microscope, camera records different concentrations changes for cell morphology in order to measure the effect of RPL36. RPL36 exhibited the high anticancer activity as can be seen from the cell morphology which was rounded

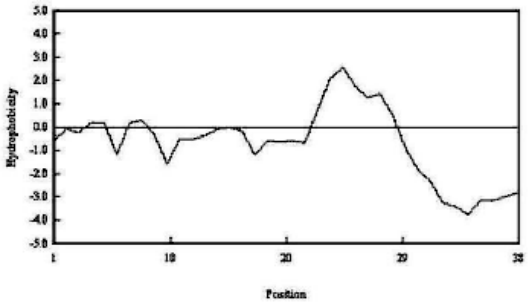

Fig. 3. The hydrophobicity analysis of RPL36 protein

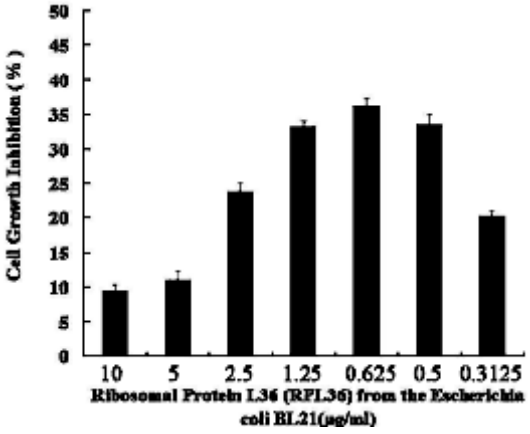

Fig.4. Cell growth inhibition of Ribosomal Protein L36 (RPL36) from the *Escherichia coli* BL21 on human laryngeal carcinoma Hep-2 cells

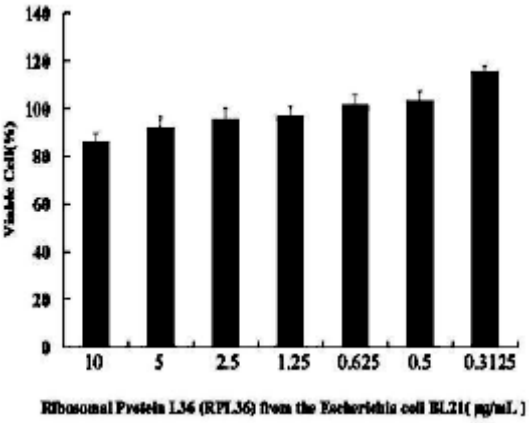

Fig. 5. Cell growth inhibition of Ribosomal Protein L36 (RPL36) from the *Escherichia coli* BL21 on human hepatoma Hep G-2 cells

into group, and even cracking off in pieces in Hep-2 group (Figure 6) while Hep G-2 cells displayed no significant change compared to those in control group (Figure 7).

RPL36 enhances cytokines productions in Hep-2

Using the ELISA method, the level of DAPK1, DAPK3 and FADD, which secreted by RPL36-effected Hep-2 cells was compared with control. Obviously, the level of these three cytokines secreted by RPL36-effected Hep-2 cells was much higher than medium-treated Hep-2 cells. It is noted that the productions of DAPK3 were stimulated at a high level when the concentration of RPL36 was only 0.625 mg/mL (Table 3).

RPL36 stimulated the expression of DAPK1, DAPK3 and FADD mRNA

Quantitative RT-PCR results showed a significant increase in the level of DAPK1, DAPK3 and FADD mRNA in RPL36-treated Hep-2 cells compared to those untreated. The expression of all the genes studied in the untreated Hep-2 cells was little, but increased dramatically in a dose-dependent manner in the RPL36-treated cells (Table 4).

Death-associated protein kinase 3 (DAPK3) induces morphological changes in apoptosis when overexpressed in mammalian cells. These results suggest that DAPK3 may play a role

Table 1. The result of primer design

Gene	Antisense (5'-3')	Sense (5'-3')	Tm (ºC)	Product Size (bp)
β-actin	GCTGTCCCTGTATGC CTCT	TTGATGTCACGCACG ATTT	55.4	222
DAPK1	GCCTTCTTGGGACTGATGCTGG	CTCTGGCTTTGTCTTTCTTGTT	51.7	385
DAPK3	GCCTATGTCTCAGCCTCT	GGTTGACTTTCTCCTGGTAT	53.4	423
FADD	GAGCGAGTTGTGGATTGTC	GGGAGGAGCTGATGGAGT	55.2	376

Table 2. Production of DAPK1, DAPK3 and FADD in Hep-2 cells stimulated by RPL36. (mean±SD,n=6)

Group	N	L1	L2	L3
DAPK1 (pg/ml)	91.3±9.2	126.4±5.3(*)	131.5±3.5(*)	144.0±4.4(*)
DAPK3 (pg/ml)	3.2±0.1	5.1±0.3(*)	9.4±0.3(**)	11.6±0.6(**)
FADD(pg/ml)	83.7±8.2	112.7±7.2(**)	124.3±5.1(**)	141.4±5.2(**)

Significant differences from negative control group and positive control group were evaluated using Student's t test: *P < 0.05, **P < 0.01.;N: negative control group; L1, L2, L3 indicating RPL36 groups of 1.25 mg/mL, 0.625mg/mL, 0.5 mg/mL, respectively.

Table 3. Expression of DAPK1, DAPK3 and FADD in Hep-2 cells stimulated by RPL36. (mean±SD,n=6)

Group	N	L1	L2	L3
DAPK1	0.03±0.01	0.43±0.04 (**)	0.87±0.22 (**)	1.01±0.18 (**)
DAPK3	0.01±0.00	0.15±0.01(**)	0.32±0.02 (**)	0.49±0.04 (**)
FADD	0.01±0.00	0.25±0.01(**)	0.45±0.03 (**)	1.02±0.08 (**)

Significant differences from negative control group and positive control group were evaluated using Student's t test: *P < 0.05, **P < 0.01.;N: negative control group; L1, L2, L3 indicating RPL36 groups of 1.25 g/mL, 0.625 g/mL, 0.5 g/mL, respectively.

in the induction of apoptosis[21]. Death-associated protein kinase 1 is an enzyme that in humans is encoded by the *DAPK1* gene. Death-associated protein kinase 1 is a positive mediator of gamma-interferon induced programmed cell death. DAPK1 encodes a structurally unique 160-kD calmodulin dependent serine-threonine kinase that carries 8 ankyrin repeats and 2 putative P-loop consensus sites. It is a tumor suppressor candidate[22]. Fas-Associated protein with Death Domain (FADD) is an adaptor molecule that bridges the Fas-receptor, and other death receptors, to caspase-8 through its death domain to form the death-inducing signaling complex (DISC) during apoptosis[23]. Our results demonstrated that RPL36 could increase the releasing of DAPK1, DAPK3 and FADD and induced expression of the three genes to several folds higher in vitro. Taken together, it is reasonable to assume that the release of DAPK1, DAPK3 and FADD of Hep-2 cells which is activated by RPL36 may be the important mechanism of the anti-tumor effect of RPL36. The results of this study support the efficacy of Escherichia coli BL21ribosomal protein L36 as an anticancer agent for human laryngeal carcinoma Hep-2 cells. However, further studies of the mechanism and the signal transduction pathways are required.

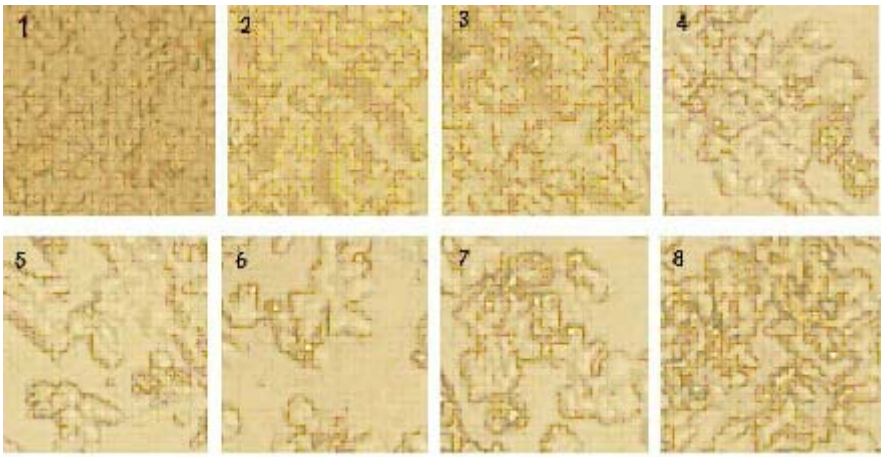

Fig. 6. The effect of Ribosomal Protein L36 (RPL36) from the *Escherichia coli* BL21 on morphology of human laryngeal carcinoma Hep-2 cells for 0, 10, 5, 2.5, 1.25, 0.625, 0.5, 0.3125µg/ml (pictures 1–8), respectively

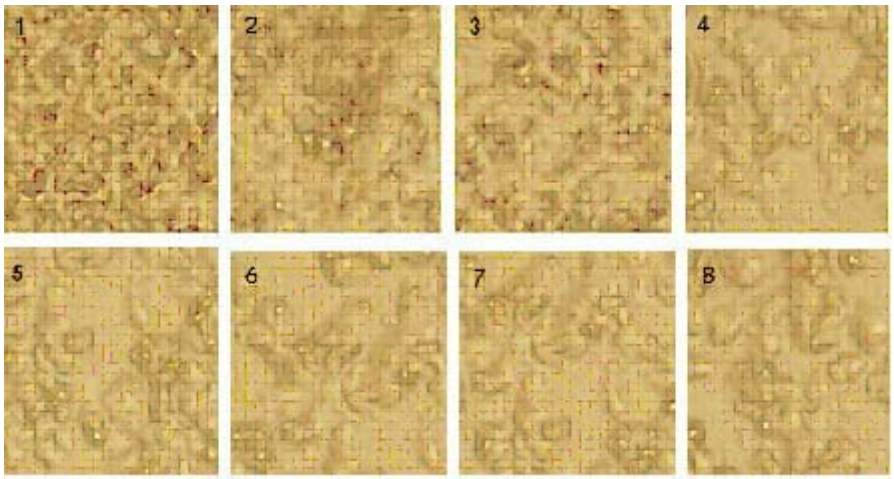

Fig. 7. The effect of Ribosomal Protein L36 (RPL36) from the *Escherichia coli* BL21 on morphology of human hepatoma Hep G-2 cells for 0, 10, 5, 2.5, 1.25, 0.625, 0.5, 0.3125µg/ml (pictures 1–8), respectively

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