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In Silico and Expression analysis of URG-4/URGCP Gene in Different Cancer Cells

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Abstract
Upregulated gene 4 (URG-4/URGCP) located in chromosome 7 (7p13), was originally suggested as one of the HBxAg upregulated genes in hepatoma cells and implicated in tumorogenesis process in different cells. In this study we analyzed URG4/URGCP expression at mRNA and protein level in various cancer cell lines namely MG-63 (Human Osteosarcoma), Saos-2 (Osteosarcoma), MCF-7 (Human Breast Carcinoma), HeLa (Human Cervical Cancer), PC3 (Human Prostate Cancer), DU-145 (Human Prostate Cancer), HT-29 (Human Colon Carcinoma), HL-60 (Human Myeloid Leukemia) and Ishikawa (Human Endometrial Adenocarcinoma). The maximum URG-4/URGCP expression was obtained in MG-63 cells (nearly 8.0 fold). It was investigated that HL-60 cells were expressed the maximum protein level of URG-4/URGCP gene (nearly two-fold). Bioinformatics analysis of URG-4/URGCP variants on the mRNA and protein sequences showed that URG-4/URGCP has 5 variants (Variant-1 (3609 bp; 922 aa), Variant-2 (3831 bp; 888 aa), Variant-3 (3036 bp; 931 aa), Variant-4 (3957 bp; 888 aa) and Variant-5 (4058 bp; 888 aa). Variant-2, -4 and -5 encode the same protein with 888 amino acids.. The expression of Variant-2 and -4 of URG-4/URGCP was detected in HT-29, PC3, DU-145, MCF-7 and Hep3B cells at mRNA level using RT-PCR strategy.
Keyword: Upregulated Gene 4/URGCP, cancer, bioinformatics.

INTRODUCTION

Cancer is fundamentally a disease of tissue growth regulation failure. In order for a normal cell to transform into a cancer cell, the genes that regulate cell growth, and differentiation must be altered. These genes are divided into two broad groups. Oncogenes are genes that promote cell growth and reproduction. Tumor suppressor genes are genes that inhibit cell division and survival. Malignant transformation can occur through the formation of novel oncogenes, the inappropriate over-expression of proto-oncogenes, or by the under-expression or disabling of tumor suppressor genes [1]. In the past decade, a very large number of proto-oncogenes and tumor-suppressor genes have been found [2]. In recent years, Upregulated gene 4 (URG4, GenBank accession no NM_017920) that located on chromosome 7 (7p13), was firstly characterized as HBx unregulated gene in hepatocellular carcinoma (HCC) and referred to as URG4 in 2002 by Satıroglu Tufan and her colleagues [3]. Later on, it was reported that overexpression of URG-4/URGCP in HepG2 and Gastric cells promoted cell growth and survival in tissue culture and nude mice, indicating this gene may be a putative oncogene that contributes importantly to multistep carcinogenesis and cell cycle regulation [3, 4]. Furthermore, it has been shown that overexpression of URG4/URGCP in HCC and gastric cancer cells upregulated cyclin D1, whereas repression of URG4/URGCP downregulated it [5]. Dodurga and his colleagues have demonstrated the URG4/URGCP gene expression in three prostate cancer cell lines (PC3, DU145 and LnCAP) [6]. The expression of URG4/URGCP gene was also found in Neuroblastoma [7], Glioblastoma [8] and osteosarcoma patients [9]. On the other hand, bioinformatics analysis suggested that there are three different protein variants translated from five different mRNA variants of URG4/URGCP gene, namely variant 1 (3609 bp; 922 aa), Variant 2 (3831 bp; 888 aa), Variant 3 (3036 bp; 931 aa), Variant 4 (3957 bp; 888 aa) and Variant 5 (4058 bp: 888 bp). However, there is no expression analysis and functional data available on the variants of URG4/URGCP gene in different cells. In addition, as a potential oncogene, the extensive expression studies of URG4/URGCP gene should be carried out in different cancer cells.

Therefore, the aim of this study could be divided into two categories; Firstly, analysis of the expression of URG4/URGCP gene at the mRNA and protein level in various cancer cell lines namely MG-63 (Human Osteosarcoma), Saos-2 (Human Osteosarcoma), MCF-7 (Human Breast Carcinoma), Hela (Human Cervical cancer, PC3 (Human Prostate cancer), HT-29 (Human Colon Carcinoma), HL-60 (Human myeloid leukemia), DU145 (Prostate cancer cells) and Ishikawa (Human endometrial adenocarcinoma); secondly bioinformatics analysis of mRNA and protein sequences of URG-4/URGCP variants and the determination of the expression of variant 2 and 4 in different cancer cells.

MATERIALS AND METHODS

Cells, Antibodies and Primers

10 different cancer cells were used in the study; The source of cells were listed as follow; MG-63 cells from Dr. Kenneth Wan (Cardiff, School of Biosciences, Cardiff UK , Saos-2 cells (Human Osteosarcoma Cell Line) from Dr. Deborah Mason (Cardiff, School of Biosciences, Cardiff UK). HeLa (Human Cervical Cancer) cells from Dr.Dipak Ramji (Cardiff, School of Biosciences, Cardiff, UK), HUVEC cells (Human Umbilical Vein Endothelial cells) from Dr. Ayşe Begüm TEKİNAY (National Nanotechnology Research Center, Bilkent University, UNAM), PC-3 and DU-145 (human prostate cancer) cells from Prof. Dr. Kemal S. Korkmaz (Ege University, Faculty of Bioengineering, İzmir, TURKEY), HL-60 (human myeloid leukemia) and HT-29 (human colon adenocarcinoma) cells from Prof. Dr. İsmet D. GÜRHAN (Ege University, Faculty of Bioengineering, İzmir, TURKEY). Ishikawa (human endometrial adenocarcinoma) cells from Prof. Dr. Ayhan Bilir (Çapa Medical Faculty, Istanbul, TURKEY), Human Pancreatic carcinoma (Panc) from ATCC. An antibody against URG-4 was purchased from Abcam (ab103323) and β-actin (sc-81178) from Sigma-Aldrich. Goat pAb to Rb IgG (ab97069) from Abcam, and Goat anti mouse IgHRP (sc-2005) were obtained from Sigma-Aldrich. Primers were obtained from Macrogen.

Cell Culture

Adherent cells were cultured in Dulbecco's modified Eagle's medium (Gibco) HL-60 suspension cells in Roswell Park Memorial Institute medium (RPMI) supplemented with 10% Fetal bovine serum (Gibco) and 2mM Glutamine in 25 cm^2 tissue culture flasks at 37 °C containing 5% CO_2.

Semi-quantitative RT-PCR

Total RNA was extracted from cultured cells using the GeneJET™ RNA Purification Kit (Thermo Scientific) according to the manufacturer's instructions. 1 µg of total RNA was reverse transcribed into complementary DNA (cDNA) using Revert Aid Reverse Transcriptase (200 U) and oligo(dT) as primer (100 pmol), (Both from Fermentas). PCR was carried out in a final volume of 50 µL in a reaction mixture containing 1 µL cDNA, 2.5 mM $MgCl_2$, 1X KCl reaction buffer, 5U/µL Taq polymerase and 100ng/µL specific primers (shown in Table 1). Reactions included with 30 cycles of denaturation at 94 °C 2 min, annealing at 55 °C for 30 s and extension at 72 °C 30 s. For normalization primers as known human β-2 microglobulin were used indicated in Table 1. The PCR condition was used: an activation step at 94 °C for 2 min, 19 cycles of 60 sec at 94°C, 30 sec at 60 °C, 45 sec at 72 °C, and a final extension step of 5 min at 72°C. Amplified products were separated by electrophoresis on a 2% agarose gel, and digital images were obtained using a Gel Doc documentation system. The intensity of images was quantified densitometric analysis using ImageJ image processing software package (ImageJ: National Institutes of Health), and normalized in respect to the corresponding fragment concentration of the ubiquitously expressed genes human β-2 microglobulin [10, 11].

Protein Extraction and Western Blot Analysis

Cells were cultured in 25 cm^2 flask/2.000.000 cells. The medium was removed, and the cells were washed with cold PBS two times and lysed in Laemli Buffer. Protein concentration was estimated using Qubit protein detection kit. 50 µg of total proteins from each extract were separated 10% SDS-polyacrylamide gel and transferred onto nitrocellulose membranes at 15V overnight. Membranes were blocked with 5% non-fat milk TBST at room temperature an hour and incubated with 1/100 anti-URG-4 (Abcam) and 1/5000 β-actin antibody (Sigma) in 5% nonfat milk in TBST an hour at room temperature. The bands were detected using an HRP-linked secondary conjugate specific for either rabbit or mouse [12].

Bioinformatics Analysis

mRNA and protein sequences of URG4/URGCP variants were obtained from NCBI database. Variants of URG4/URGCP genes were compared using BioEdit v.7.2.5 programme. Predicted 3D structure models of URG4/URGCP protein was obtained using I-TASSER programme (http://zhanglab.ccmb.med.umich.edu/I-TASSER/).

RESULTS

***In silico* Analysis of URG-4/URGCP Gene**

URG-4/URGCP gene was located on chromosome 7 and 50 000 bp long with five introns and six exons. Bioinformatics analysis showed that there are three different protein variants originated from 5 different mRNAs. The accession number of mRNA of these variants are as follows; Variant 1 (3609 bp; 922 aa) NM_017920, Variant 2 (3831 bp; 888 aa) NM_001077664, Variant 3 (3036 bp; 931 aa) NM_001077663, Variant-4 (3957 bp; 888 aa) NM_001290076 and Variant 5 (4058 bp; 888 bp) NM_001290075.1. Although Variant 4 of URG-4/URGCP has the longest mRNA sequence due to the long 5' UTR, the longest protein is Variant 3 with 931 aa. The variant 4 differs in the 5' UTR, lacks a portion of the 5' coding region and initiates translation from downstream start codon compared to Variant 3. The encoded protein of the variant 2 (888 amino acids) has a shorter N-terminus compared to variant 3. Variant 2, 4 and 5 encode the same protein with 888 amino acids (Figure 1A).

Table 1. List of the primers

Name	Sequence (5'-3')	Tm (°C)
URG-4 F	CTTCATCCTGAGTCCCTACCG	54
URG-4 R	GCCGTTCTGCTGCATTCG	47
Human β-2 microglobulin F	TTTCTGGCCTGGAGGCTATC	51
Human β-2 microglobulin R	CATGTCTCGATCCCACTTAACT	54
URG-4 Var 2 /4 F	CAGAGTTAAGAACCACTATTCTCAGGAGTT	70
URG-4 Var 2 /4 R	CTTCGACCACTAGTCAGCAAGGCC	62

A

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Var 1   1   ---------MASPGHSDLGEVAPEIKASERRTAVAIADLEWREMEGDDCEFRYGDGTNEAQDNDFPTVERSRLQEMLSLLGLETYQVQKLSLQDSLQISF 91
Var 3   1   MASPGIEVELLGKGHSDLGEVAPEIKASERRTAVAIADLEWREMEGDDCEFRYGDGTNEAQDNDFPTVERSRLQEMLSLLGLETYQVQKLSLQDSLQISF 100
Var 2   1   ----------------------------------------------MEGDDCEFRYGDGTNEAQDNDFPTVERSRLQEMLSLLGLETYQVQKLSLQDSLQISF 57
Var 4   1   ----------------------------------------------MEGDDCEFRYGDGTNEAQDNDFPTVERSRLQEMLSLLGLETYQVQKLSLQDSLQISF 57
Var 5   1   ----------------------------------------------MEGDDCEFRYGDGTNEAQDNDFPTVERSRLQEMLSLLGLETYQVQKLSLQDSLQISF 57

Var 1   92  DSMKNWAPQVPKDLPWNFLRKLQALNADARNTTMVLDVLPDARPVEKESQMEEEIIYWDPADDLAADIYSFSELPTPDTPVNPLDLLCALLLSSDSFLQQ 191
Var 3   101 DSMKNWAPQVPKDLPWNFLRKLQALNADARNTTMVLDVLPDARPVEKESQMEEEIIYWDPADDLAADIYSFSELPTPDTPVNPLDLLCALLLSSDSFLQQ 200
Var 2   58  DSMKNWAPQVPKDLPWNFLRKLQALNADARNTTMVLDVLPDARPVEKESQMEEEIIYWDPADDLAADIYSFSELPTPDTPVNPLDLLCALLLSSDSFLQQ 157
Var 4   58  DSMKNWAPQVPKDLPWNFLRKLQALNADARNTTMVLDVLPDARPVEKESQMEEEIIYWDPADDLAADIYSFSELPTPDTPVNPLDLLCALLLSSDSFLQQ 157
Var 5   58  DSMKNWAPQVPKDLPWNFLRKLQALNADARNTTMVLDVLPDARPVEKESQMEEEIIYWDPADDLAADIYSFSELPTPDTPVNPLDLLCALLLSSDSFLQQ 157

Var 1   192 EIALKMALCQFALPLVLPDSENHYHTFLLWAMRGIVRTWWSQPPRGMGSFREDSVVLSRAPAFAFVRMDVSSNSKSQLLNAVLSPGHRQWDCFWHRDLNL 291
Var 3   201 EIALKMALCQFALPLVLPDSENHYHTFLLWAMRGIVRTWWSQPPRGMGSFREDSVVLSRAPAFAFVRMDVSSNSKSQLLNAVLSPGHRQWDCFWHRDLNL 300
Var 2   158 EIALKMALCQFALPLVLPDSENHYHTFLLWAMRGIVRTWWSQPPRGMGSFREDSVVLSRAPAFAFVRMDVSSNSKSQLLNAVLSPGHRQWDCFWHRDLNL 257
Var 4   158 EIALKMALCQFALPLVLPDSENHYHTFLLWAMRGIVRTWWSQPPRGMGSFREDSVVLSRAPAFAFVRMDVSSNSKSQLLNAVLSPGHRQWDCFWHRDLNL 257
Var 5   158 EIALKMALCQFALPLVLPDSENHYHTFLLWAMRGIVRTWWSQPPRGMGSFREDSVVLSRAPAFAFVRMDVSSNSKSQLLNAVLSPGHRQWDCFWHRDLNL 257

Var 1   292 GTNAREISDGLVEISWFFPSGREDLDIFPEPVAFLNLRGDIGSHWLQFKLLTEISSAVFILTDNISKKEYKLLYSMKESTTKYYFILSPYRGKRNTNLRF 391
Var 3   301 GTNAREISDGLVEISWFFPSGREDLDIFPEPVAFLNLRGDIGSHWLQFKLLTEISSAVFILTDNISKKEYKLLYSMKESTTKYYFILSPYRGKRNTNLRF 400
Var 2   258 GTNAREISDGLVEISWFFPSGREDLDIFPEPVAFLNLRGDIGSHWLQFKLLTEISSAVFILTDNISKKEYKLLYSMKESTTKYYFILSPYRGKRNTNLRF 357
Var 4   258 GTNAREISDGLVEISWFFPSGREDLDIFPEPVAFLNLRGDIGSHWLQFKLLTEISSAVFILTDNISKKEYKLLYSMKESTTKYYFILSPYRGKRNTNLRF 357
Var 5   258 GTNAREISDGLVEISWFFPSGREDLDIFPEPVAFLNLRGDIGSHWLQFKLLTEISSAVFILTDNISKKEYKLLYSMKESTTKYYFILSPYRGKRNTNLRF 357

Var 1   392 LNKLIPVLKIDHSHVLVKVSSTDSDSFVKRIRAIVGNVLRAPCRRVSVEDMAHAARKLGLKVDEDCEECQKAKDRMERITRKIKDSDAYRRDELRLQGDP 491
Var 3   401 LNKLIPVLKIDHSHVLVKVSSTDSDSFVKRIRAIVGNVLRAPCRRVSVEDMAHAARKLGLKVDEDCEECQKAKDRMERITRKIKDSDAYRRDELRLQGDP 500
Var 2   358 LNKLIPVLKIDHSHVLVKVSSTDSDSFVKRIRAIVGNVLRAPCRRVSVEDMAHAARKLGLKVDEDCEECQKAKDRMERITRKIKDSDAYRRDELRLQGDP 457
Var 4   358 LNKLIPVLKIDHSHVLVKVSSTDSDSFVKRIRAIVGNVLRAPCRRVSVEDMAHAARKLGLKVDEDCEECQKAKDRMERITRKIKDSDAYRRDELRLQGDP 457
Var 5   358 LNKLIPVLKIDHSHVLVKVSSTDSDSFVKRIRAIVGNVLRAPCRRVSVEDMAHAARKLGLKVDEDCEECQKAKDRMERITRKIKDSDAYRRDELRLQGDP 457

Var 1   492 WRKAAQVEKEFCQLQWAVDPPEKHRAELRRRLLELRMQQNGHDPSSGVQEFISGISSPSLSEKQYFLRWMEWGLARVAQPRLRQPPETLLTLRPKHGGTT 591
Var 3   501 WRKAAQVEKEFCQLQWAVDPPEKHRAELRRRLLELRMQQNGHDPSSGVQEFISGISSPSLSEKQYFLRWMEWGLARVAQPRLRQPPETLLTLRPKHGGTT 600
Var 2   458 WRKAAQVEKEFCQLQWAVDPPEKHRAELRRRLLELRMQQNGHDPSSGVQEFISGISSPSLSEKQYFLRWMEWGLARVAQPRLRQPPETLLTLRPKHGGTT 557
Var 4   458 WRKAAQVEKEFCQLQWAVDPPEKHRAELRRRLLELRMQQNGHDPSSGVQEFISGISSPSLSEKQYFLRWMEWGLARVAQPRLRQPPETLLTLRPKHGGTT 557
Var 5   458 WRKAAQVEKEFCQLQWAVDPPEKHRAELRRRLLELRMQQNGHDPSSGVQEFISGISSPSLSEKQYFLRWMEWGLARVAQPRLRQPPETLLTLRPKHGGTT 557

Var 1   592 DVGEPLWPEPLGVEHFLREMGQFYEAESCLVEAGRLPAGQRRFAHFPGLASELLLTGLPLELIDGSTLSMPVRWVTGLLKELHVRLERRSRLVVLSTVGV 691
Var 3   601 DVGEPLWPEPLGVEHFLREMGQFYEAESCLVEAGRLPAGQRRFAHFPGLASELLLTGLPLELIDGSTLSMPVRWVTGLLKELHVRLERRSRLVVLSTVGV 700
Var 2   558 DVGEPLWPEPLGVEHFLREMGQFYEAESCLVEAGRLPAGQRRFAHFPGLASELLLTGLPLELIDGSTLSMPVRWVTGLLKELHVRLERRSRLVVLSTVGV 657
Var 4   558 DVGEPLWPEPLGVEHFLREMGQFYEAESCLVEAGRLPAGQRRFAHFPGLASELLLTGLPLELIDGSTLSMPVRWVTGLLKELHVRLERRSRLVVLSTVGV 657
Var 5   558 DVGEPLWPEPLGVEHFLREMGQFYEAESCLVEAGRLPAGQRRFAHFPGLASELLLTGLPLELIDGSTLSMPVRWVTGLLKELHVRLERRSRLVVLSTVGV 657

Var 1   692 PGTGKSTLLNTMFGLRFATGKSCGPRGAFMQLITVAEGFSQDLGCDHILVIDSGGLIGGALTSAGDRFELEASLATLLMGLSNVTVISLAETKDIPAAIL 791
Var 3   701 PGTGKSTLLNTMFGLRFATGKSCGPRGAFMQLITVAEGFSQDLGCDHILVIDSGGLIGGALTSAGDRFELEASLATLLMGLSNVTVISLAETKDIPAAIL 800
Var 2   658 PGTGKSTLLNTMFGLRFATGKSCGPRGAFMQLITVAEGFSQDLGCDHILVIDSGGLIGGALTSAGDRFELEASLATLLMGLSNVTVISLAETKDIPAAIL 757
Var 4   658 PGTGKSTLLNTMFGLRFATGKSCGPRGAFMQLITVAEGFSQDLGCDHILVIDSGGLIGGALTSAGDRFELEASLATLLMGLSNVTVISLAETKDIPAAIL 757
Var 5   658 PGTGKSTLLNTMFGLRFATGKSCGPRGAFMQLITVAEGFSQDLGCDHILVIDSGGLIGGALTSAGDRFELEASLATLLMGLSNVTVISLAETKDIPAAIL 757

Var 1   792 HAFLRLERTGHMPNYQFVYQNLHDVSVPGPRPRDKRQLLDPPGDLSRAAAQMEKQGDGFRALAGLAFCDPEKQHIWHIPGLWHGAPPMAAVSLAYSEAIF 891
Var 3   801 HAFLRLERTGHMPNYQFVYQNLHDVSVPGPRPRDKRQLLDPPGDLSRAAAQMEKQGDGFRALAGLAFCDPEKQHIWHIPGLWHGAPPMAAVSLAYSEAIF 900
Var 2   758 HAFLRLERTGHMPNYQFVYQNLHDVSVPGPRPRDKRQLLDPPGDLSRAAAQMEKQGDGFRALAGLAFCDPEKQHIWHIPGLWHGAPPMAAVSLAYSEAIF 857
Var 4   758 HAFLRLERTGHMPNYQFVYQNLHDVSVPGPRPRDKRQLLDPPGDLSRAAAQMEKQGDGFRALAGLAFCDPEKQHIWHIPGLWHGAPPMAAVSLAYSEAIF 857
Var 5   758 HAFLRLERTGHMPNYQFVYQNLHDVSVPGPRPRDKRQLLDPPGDLSRAAAQMEKQGDGFRALAGLAFCDPEKQHIWHIPGLWHGAPPMAAVSLAYSEAIF 857

Var 1   892 ELKRCLLENIRNGLSNQNKNIQQLIELVRRL 922
Var 3   901 ELKRCLLENIRNGLSNQNKNIQQLIELVRRL 931
Var 2   858 ELKRCLLENIRNGLSNQNKNIQQLIELVRRL 888
Var 4   858 ELKRCLLENIRNGLSNQNKNIQQLIELVRRL 888
Var 5   858 ELKRCLLENIRNGLSNQNKNIQQLIELVRRL 888
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B

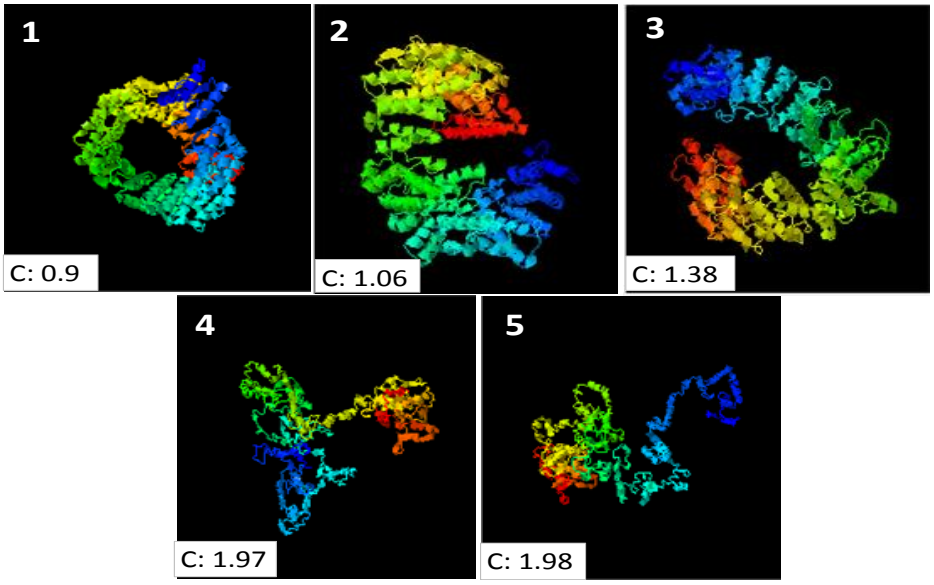

Fig 1. *In silico* analysis of URG-4/URGCP variants. **A.** Comparing the sequence of four URG4/URGCP variants using Bioedit program**. B.** Five different 3D structure protein model of Variant 1 protein of URG-4/URGCP were predicted using I-TASSER programme.

There is limited information on the cellular function of the URG-4/URGCP protein. Therefore, we used web-based tool namely I-TASSER for searching the structure and functional data for URG-4/URGCP protein. I-TASSER is a hierarchical method for protein structure and function prediction. Structural templates are first identified from the PDB by multiple threading approach LOMETS; full-length atomic models are constructed by iterative template fragment assembly simulations; function insights of the target are finally derived by threading the 3D models through protein function database. For each target, I-TASSER simulations generate tens of thousands

conformations. To select the final models, I-TASSER uses SPICKER program to cluster all the decoys based on the pair-wise structure similarity, and report up to five models which correspond to the five largest structure clusters. In Monte Carlo theory, the largest clusters correspond to the states of the largest partition function (or lowest free energy) and, therefore, have the highest confidence. The confidence of each model is quantitatively measured by C-score. Since the top 5 models are ranked by the cluster size, it is possible that the lower-rank models have a higher C-score. C-score is a confidence score for estimating the quality of predicted models by I-TASSER. It is calculated based on the significance of threading template alignments and the convergence parameters of the structure assembly simulations. C-score is typically in the range of [-5,2], where a C-score of higher value signifies a model with a high confidence and vice-versa [13-15]. The model 5 shows the highest C score with 1.98. In our results, I-TASSER programme suggested five 3D structure models for URG-4/URGCP variant -1 protein (Figure 1B). I-TASSER analyses showed that URG-4/URGCP protein was similar with Human Guanylate Binding protein.

Expression Analysis of Variant 2 and 4 of URG-4/URGCP in Different Cancer Cells

Five variants of URG-4 mRNA sequences were compared using BioEdit v.7.2.5 programme. The first exon of Variant-2 and -4 were specific and different from the other variants as shown in figure 2A. We designed specific primers amplifying only Variant-2 and -4 mRNA sequences in order to investigate the expression of these variants in different cancer cell lines (Figure 2B). Expression of Variant -2 and -4 URG-4/URGCP genes was shown in HT-29, PC3, DU-145, MCF-7 and Hep3B cells at mRNA level using RT-PCR strategy (Figure 2B). PCR Images were visualized using Gel Doc documentation system in 2% agarose gel. mRNA expression of URG-4/URGCP variant 2 and 4 was present in all cancer lines investigated.

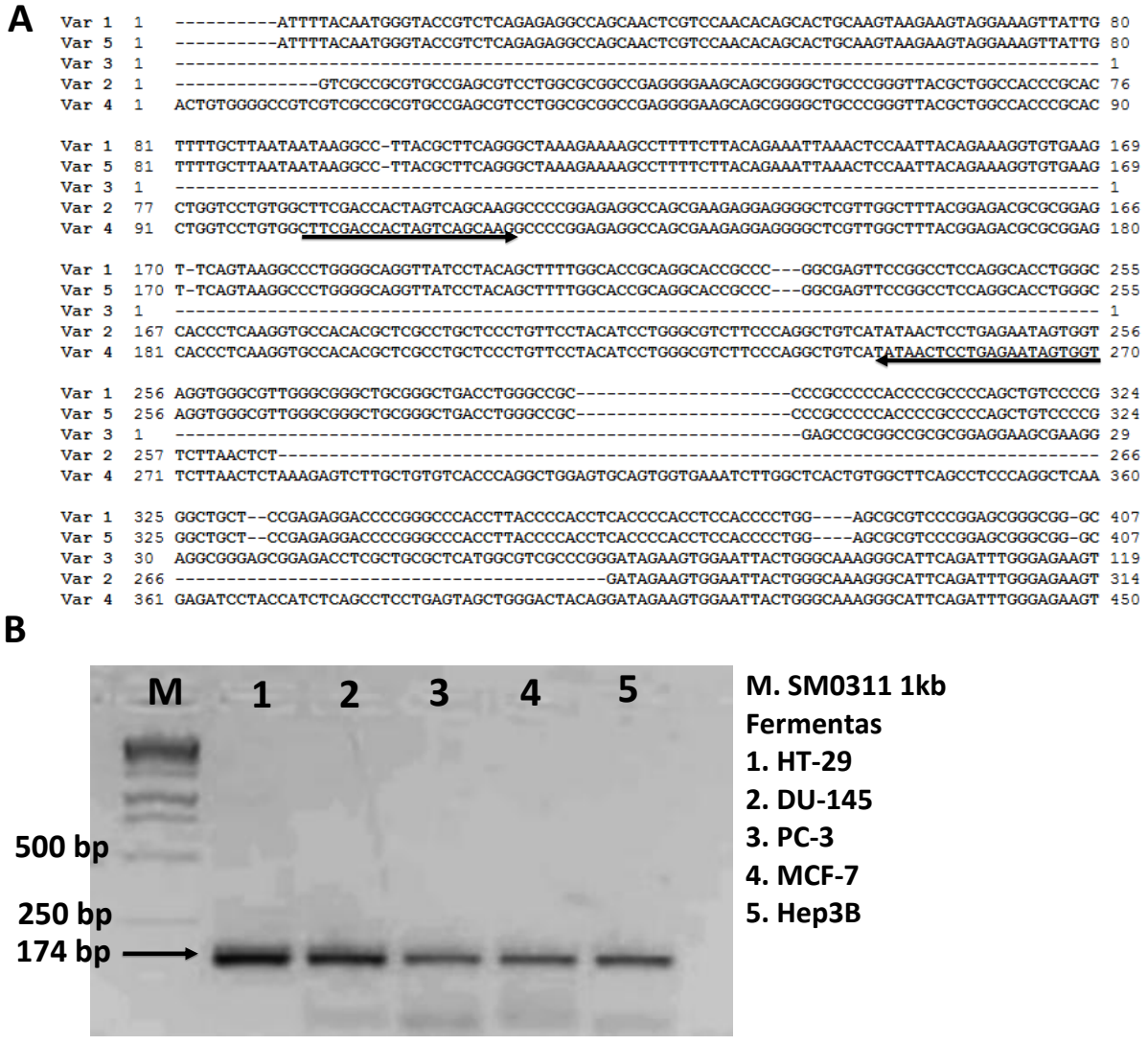

Fig 2. A. Comparing mRNA sequences of Variant -2 and -4 of URG-4/URGCP using Bioedit program. The primers specific for Variant -2 and -4 are indicated as Black arrows. **B.** The expression of Variant -2 and - 4 in different cancer cells at mRNA level. SqRT-PCR was performed specific primers of Variant -2 and -4 mRNAs.

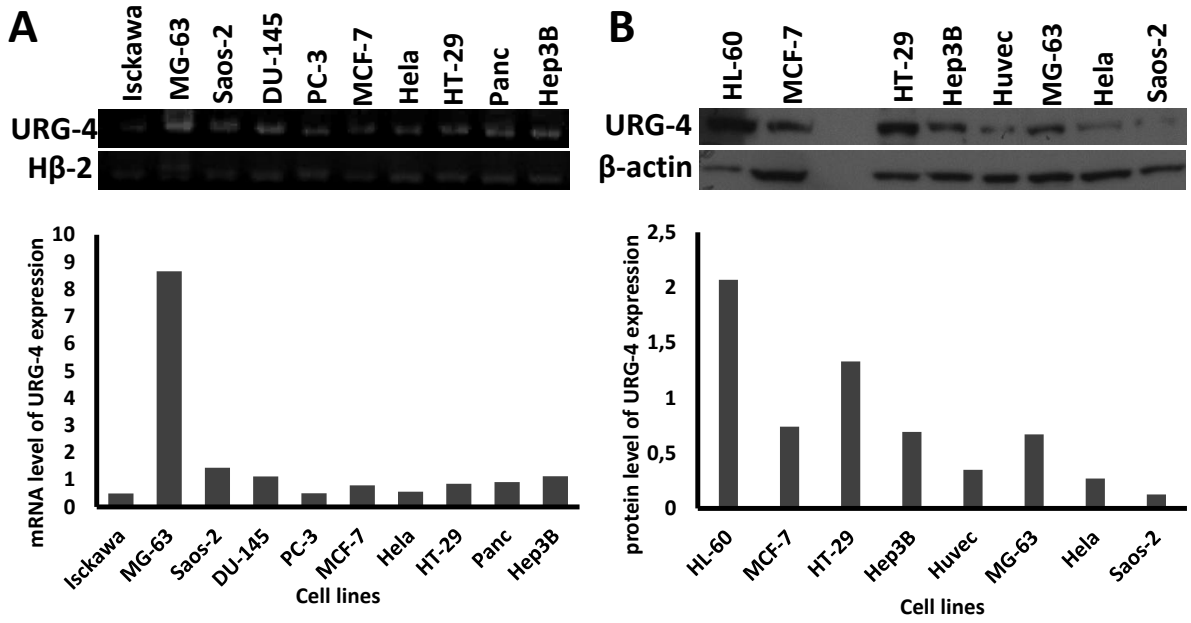

Fig 3. The expression profile of URG-4/URGCP gene at mRNA and protein level in different cancer cell lines. **A.** mRNA level of URG-4 expression was determined using Sq RT-PCR with primers specific to all URG-4/URGCP variants as described in material method section. For normalization, human β-2 primers were used as an internal control. Densitometry analyses were carried out using IMAGE J programme. Fold mRNA level was obtained by the values of URG-4/URGCP expression divided into Hβ-2 expression values. **B**. The protein level of URG-4 expression was determined using Western blotting method. 1/100 dilution of antiURG4/URGCP antibody and 1/5000 dilution of Anti β-actin antibody were used to obtain specific bands. 103 kDa URG-4/URGCP bands and 44 kDa β-actin bands were visualized with polyclonal HRP conjugated anti-rabbit and anti-mouse antibodies. The bands were analyzed with IMAGE J programme.

Analyses of URG-4 Expression at mRNA and Protein Level in Different Cancer Cells

The expression of URG-4 at mRNA level was determined in different Cancer cells namely Human endometrial adenocarcinoma (Ishikawa), Human osteosarcoma cell lines (MG-63 and Saos-2), Human Cervical Cancer cell line (Hela), Human colon adenocarcinoma (HT-29), Human prostate cancer cell lines (PC3 and DU-145), human breast adenocarcinoma (MCF-7), Human Hepatoma cell line (Hep3B) and Human Pancreatic Carcinoma (Panc)using RT-PCR strategy. Sq-RT-PCR analysis was performed as described in the material method section with specific primers to URG-4/URGCP all variants. To normalize URG-4 expression, Hβ-2 primers were used as internal control. Densitometry analysis was performed using Image J (NIH) programme and normalized to Hβ-2. Our results showed that, the maximum URG-4/URGCP expression was obtained in MG-63 cells (nearly 8.0 fold). (Figure 3A). In addition, we investigated protein level of URG-4/URGCP gene using western blotting method as described in material-method section. We used 50 µg of HL-60, MCF-7, HT-29, Hep3B, Huvec, MG-63, Saos-2 and Hela protein extracts. Human myeloid leukemia (HL-60) had the maximum protein level of URG-4/URGCP nearly two-fold when compared the others.

DISCUSSIONS

URG-4/URGCP gene has been identified in Hepatitis B infected liver and Hepatocellular Carcinoma cells where it contained with HBxAg and was weakly expressed in the uninfected liver by Satıroglu Tufan in 2002 [3]. Previous studies suggested that it may be involved in cell cycle progression through the regulation of cyclin D1 expression. Also, over-expression of URG4 in HepG2 and GES-1 cells promoted cell growth and survival in tissue culture and nude mice, indicating this gene may be associated with the onset of tumorigenesis.

URG-4/URGCP has five variants that has no information about the roles in cells. We compared the differences of mRNA and protein sequences of all variants using Bioedit V 7.2.5 programme. Variant 2, 4 and 5 encode the same protein sequence that 888 amino acids. In order to evaluate if there is the specific expression of variant 2 and 4 in different cancer cells, the expression of URG-4/URGCP variant 2 and 4 were investigated in some cancer cell lines, namely HT-29, DU-145, PC-3, MCF-7 and Hep3B cells. We found that variant 2 and variant 4 are very well expressed in these cells. However, there might be several questions arising about the differences in the expression of all variants. In addition, because of limited information of protein structure of URG-4/URGCP, ITASSER analyses were carried out for search protein structure of URG-4/URGCP protein variant 1. ITASSER programme predicted five different 3D structure with calculating C score which based on the relative clustering structural density and the consensus significance score of multiple threading templates is introduced to estimate the accuracy of the I-TASSER predictions. In our results showed that The fifth 3D model is close to URG-4/URGCP variant-1 protein with 1.98 C score.

To date, the expression of URG-4 was shown in Human Hepatoma cell lines (HepG2 and Hep3B), Human prostate carcinoma cells (PC3, DU-145 and LnCap), Gastric Cancer cells (GES-1) and CCRF-CEM (ALL-T cells)[16]. In addition, it was investigated that URG-4/URGCP was highly expressed in glioblastoma, ovarium and neuroblastoma tissues. This was the first time to investigate the comparative analyses of expression URG-4/URGCP gene nearly ten different cells lines. Our results

showed that URG-4/URGCP gene was expressed in all cancer cells at mRNA level. Interestingly MG-63 osteosarcoma cells were expressed the maximum mRNA level of this gene (nearly eight-fold). However, Saos-2 cells had URG-4/URGCP expression low level (nearly two-fold. Previously a study showed that Saos-2 cells exhibit a more mature osteoblast phenotype, compared with that of MG63 cells, rendering them a good candidate for an *in vitro* model of osseointegration [17]. Probably, high expression level of URG-4/URGCP gene in MG-63 cells correlated with being not mature. In addition, URG-4/URGCP gene interestingly expressed nearly ten different cancer cell lines, showed that this gene really may be a potential oncogene. Also, previous studies indicated that over expression of this gene promoted cell growth and survival, suggesting that URG-4/URGCP may be associated with the development of carcinogenesis.

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