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Construction of expressing vectors including melanoma differentiation-associated gene-7 (mda-7) fused with the RGD sequences for better tumor targeting

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ABSTRACT

Objective(s): Up to now, many researches have been performed to improve the antitumoral effect of melanoma differentiation-associated gene-7 (mda-7) protein. The purpose of our research was to construct 3 expression vectors producing mda-7 in fusion with RGD (Arginine-Glycine-Aspartic acid) peptide and evaluate their expression.

Materials and Methods: mda-7 gene with two different RGD sequences was amplified by PCR then was cloned by TA–cloning system. The colonies including these genes were selected by blue–white screening, colony PCR, and sequencing, respectively. Afterward, the genes were sub-cloned into the expression vector following confirmation by colony PCR and sequencing. In addition, these constructs were transfected into 293 and Huh-7 cells for further expression analysis. The mda-7 gene expression was evaluated by RT-PCR and IF (immunofluorescence assay). DNA laddering test and trypan blue exclusion assays were performed to screen cytotoxicity of prepared plasmids.

Results: Three different mda-7 genes with terminal RGD peptide were cloned correctly into the expression vectors and their expression was confirmed to be suitable by RT-PCR and IF assay. It was shown that expressions were limited to those transfected, GFP shining cells. No significant cytotoxicity was observed by simple assays in all plasmid treated cells. In expressing cells, all forms of mda-7 protein were localized mainly around ER prenuclear compartment while GFP protein was distributed evenly among them.

Conclusion: Theoretically RGD tagged mda-7 would be able to induce apoptosis with more specificity and stronger than the standard one, therefore, these new constructs may have the potential for further researches.

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Introduction

Melanoma differentiation-associated gene-7 (mda-7) is a novel tumor suppressor gene first identified using subtraction hybridization method by Fisher in 1995 (1). Based on its cytokine-like properties, structure, and chromosomal location, mda-7 has been renamed to interleukin 24 (IL-24) and classified as a member of interleukin 10 (IL-10) gene family (2). Subsequent analysis revealed its growth inhibitory and apoptotic induction in a broad spectrum of human cancer cells without excreting harmful effects on normal cells; therefore, has received considerable attention for more therapeutic application in the field of cancer (3-10). This interleukin exerts its antitumor functions through multitude of pathways, both intracellular and exogenously as well.

Secreted mda-7/IL-24, like other cytokines acts through IL-22R1/IL-20R2 and IL-20R1/IL20R2 receptors and JAK/STAT pathway (11). The activated STAT by binding of exogenous mda-7 to cognate receptors induces BAX protein expression which finally improves death signal. Furthermore, mda-7/IL-24 protein up-regulates its own expression level in a positive-feedback loop upon stabilizing of its mRNA structure (12). As a secreted cytokine the "potent bystander apoptosis-inducing effect" phenomena has been well documented in mda-7/IL-24 gene therapies based on these two mentioned pathways (13).

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Yet to reap the full reward of its deployment as a therapeutic anticancer gene, further studies are performing to enhance its apoptosis inducing features by employing more efficient delivery strategies (14). Over expression of αvβ3 and αvβ5 integrin has been well documented in a wide variety of tumor cell lines (10, 15-17). The exposed RGD motif within extracellular matrix proteins is considered as the most relevant biding site for integrin receptors. It has been demonstrated that ligand binding specificity and activity of these receptors is highly influenced by RGD adjacent residues within the protein structure (18).

The previous studies have shown improving effect of therapeutic cytokines by joining with RGD motif especially in tumor models (19-21). Fusion of cytokines with RGD motif makes them more concentrated in tumor microenvironment enriched by integrin ligand and consequently boosts their effectiveness through specific attachment to tumor cells (19-21). To our knowledge, limited number of studies have investigated joining RGD or related targeting peptide to mda-7 for gene therapeutic approaches (20) while other related experiments have introduced RGD targeting strategy for protein based therapy (19, 22). Here, in order to enhance mda-7/IL-24 cell adhesion rather than elevating its inherent apoptotic induction through improving the bystander effect, two kinds of RGD motif is introduced at the end of mda-7/IL-24 structure. Finally, the expression of these modified mda-7 proteins by eukaryotic expression vector is evaluated in human normal and tumor lines.

Materials and Methods

Reagents

Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), and lipofectamine 2000 transfection reagent were purchased from Invitrogen, USA. Penicillin, streptomycin, and trypsin were purchased from Sigma Co, Germany. Total RNA isolation and cDNA synthesis kits were obtained from Bioneer (South Korea). Other molecular reagents were purchased from CinnaGen Co (Iran) and Bioneer, (South Korea). All antibodies were purchased from R&D Co, (USA).

Cell lines and cell culture

Human cell lines including HEK-293 (normal embryonic cell line) and Huh-7 (hepatocellular tumor line) were purchased from national cell bank (Pasteur Institute of Iran) and were maintained in complete DMEM supplemented with 10% FBS and penicillin/streptomycin mixture at 37 ˚C in a humidified atmosphere of 5% CO_2. Cells at logarithmic phase of growth were harvested with 0.25% trypsin (19).

Amplification of the mda-7 and mda-7-RGD sequences

The original mda-7 plasmid was a gift from Dr Stephanie Kreis from "Laboratoire de Biologie et Physiologie Integree (LBPI)", University of Luxembourg as described before (23). The primers used for PCR reaction were designed by online primer designer deposited in NCBI, based on the original mda-7/IL-24 sequence. While designing the primers, two different cyclic RGD peptides in addition to BglII restriction site were introduced at primer ends as listed in Table 1. Our selected smaller version of RGD motifs (**CD<u>CDGRC</u>FC**), has little difference from original cyclic RGD-4C (A**C**D**<u>CRGDC</u>**F**C**G), as two terminal amino-acids were deleted (Figure 1). Theoretically larger size of original RGD sequence may induce immune response in host body which will be alleviated in smaller RGD version that was selected here (21).

The PCR amplification for standard mda-7 gene was performed by using MD1 as forward and MD2 as reverse primers. Amplification of mda-7 with terminal RGD peptide sequences was performed using MD1 as forward and RGD1 or RGD2 as reverse primers. 27 cycles of touchdown PCR reaction with the temperature range at 63-53 degrees was developed for large scale amplification of these sequences by including 1.5 unit of Max Taq enzyme (polymerase enzyme blend from Vivantis, Malaysia), 0.2 µm of each primers, and 100 ng linear plasmid containing mda-7 in reaction tubes.

Cloning the genes into expression vector

At first, proper PCR amplicons were extracted from the agarose gel and ligated into *pTZ57R/T* cloning vector (InsTAclone PCR cloning Kit, Fermentas Co, Canada) using T4 DNA ligase. Subsequent transformation was performed in competent DH5α bacteria via the heat shock method. Finally, the true transformed colonies were selected through

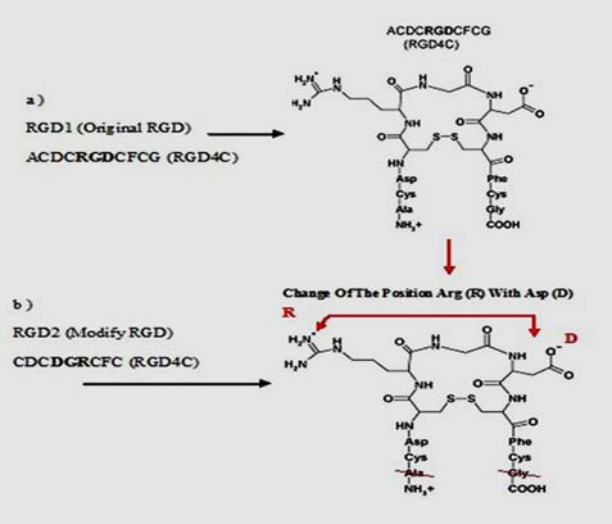

Figure 1. Chemical structures of, a) RGD1 (original RGD4C) (24) and b) RGD2 (modified RGD4C)

Table 1. The primer sequences used for amplification of different mda-7 genes by PCR reaction. The site of restriction enzyme BglII has been underlined and the sequence for RGD motif has been shown in Italic format

Primer	Sequence (5' → 3')	Fragment (bp)
MD1	GGAAGATCTACCATGAATTTTCAACAG	649
MD2	GGAAGATCTGTCGACTCAGAGAGCTTGTAGAATT	649
RGD1	GGAAGATCTTCAGCAGAA*GCAGTCACCACGGCA*GTCGCAGAGCTTGTAGAATT	702
RGD2	GGAAGATCTTCA*GCAACGACCGTCGCA*GAGCTTGTAGAATT	690
CMV1	TGTGGCAGGCTTGAGATCCAGCT	~ 10300
RT1	GGGCTGTGAAAGACACTAT	381
RT2	GCATCCAGGTCAGAAGAA	
GAP-F	GAA GGT GAA GGT CGG AGT C	202
GAP-R	GAA GAT GGT GAT GGG ATT TC	

blue–white screening assay on LB agar containing 50 µg/ml ampicillin, colony-PCR, and sequencing methods.

The positive colonies which were obtained from colony-PCR assay were digested by restriction enzyme BglII then proper fragments of suitable sequences (nominated as mda-7, mda-7-RGD1, and mda-7-RGD2) were extracted from the agarose gel electrophoresis.

On the other hand, eukaryotic expression vector (*pAdenoVator-CMV5-IRES-GFP*) was digested with BglII and extracted from agarose gel following dephosphorylation with calf intestinal alkaline phosphatase. This dephosphorylated vector and desired digested fragments were ligated together by T4 DNA ligase enzyme in 1x ligase buffer while incubated at room temperature overnight. Transformation was performed in competent bacteria of DH5α and colonies were grown on LB agar containing 75 µg/ml kanamycin for screening following colony-PCR and sequencing as next confirmation steps.

The right orientation of true transformed colonies was screened by a PCR-colony assay performed for 35 cycles using CMV1 primer as forward and MD2, RGD1 or RGD2 as reverse primers. For more confirmation, separate digestion assays using BstXI and BglII enzymes were performed to evaluate the integrity of constructs. Furthermore direct sequencing of inserted fragments by employing common primers was performed as final confirmatory step.

Afterward, true plasmids designated as pIRES/mda-7, pIRES/mda-7-RGD1, and pIRES/mda-7-RGD2 were extracted according to the manufacturer's instructions with miniprep kit (Invitek Co, Germany).

DNA transfection assays

HEK-293 and Huh-7 cells were cultured in 24 wells plate, 24 hr before transfection. There were totally 10^5 cells in each well transfected by eukaryotic expression vector using lipofectamine 2000 transfection reagent according to manufacturer's protocol (19). Briefly, the cell lines were transfected with a complex of appropriate amount of lipofectamine 2000 and 500 ng of each expression plasmids, then leaving them 4 hr followed by addition of complete DMEM medium and further incubation at 37 ˚C for 24 hr.

Direct fluorescence assay, trypan blue exclusion assay, and laddering test

In cells transfected with expression plasmid, the GFP fluorescence was observed using a direct fluorescence microscope (Nikon fluorescent microscopy). The trypan blue exclusion assay was performed to screen significant cytotoxicity of the plasmids in Huh-7 and HEK-293 cells over 24 hr. For this purpose transfected cells were harvested and diluted at a concentration of 10^5 cells/ml, then 50 µl of this volume was mixed with equal volume of trypan blue dye. The dead versus live cells were counted on glass slide then final percentage was calculated per 100 cell counts. DNA fragmentation assay was also performed as described previously (25) in except that total DNA was extracted by DNP™ kit from Cinnaclone, Iran. Furthermore, untransfected cells served as negative control in both tests.

RNA isolation and RT-PCR

Total cellular RNA was extracted from cells at 24 hr post transfection using the RNX–Plus kit from CinnaGen Co. (Iran), according to the manufacturer's instructions. Residual DNA fragments were removed by DNase enzyme (Promega Co, USA) and RT-PCR was performed by cDNA synthesis kit. New primer pair (RT1 and RT2) was designed based on the reported IL-24 cDNA sequence for better detection and quantification of gene expression *in vitro* as shown in Table 1.

Reaction program with described primers was run as follows: denaturation at 95 ˚C for 3 min; 30 cycles at 94 ˚C for 30 sec, 55 ˚C for 30 sec, and 72 ˚C for 30 sec. The amplified products were visualized on 1% agarose gel electrophoresis. The GAPDH (Glyceraldehyde 3-phosphate dehydrogenase) gene was also included as internal positive control to validate all the procedures.

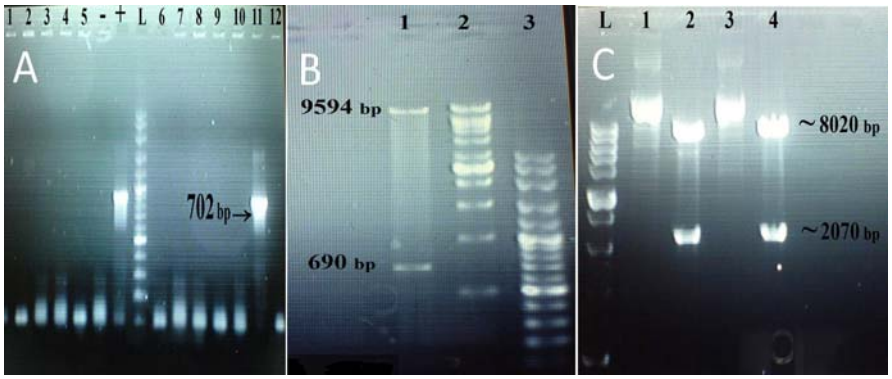

Figure 2. Molecular steps for confirmation of cloning and vectors integrity. A. The positive bacterial colonies were selected by colony-PCR. The positive (1/10 dilution of ligation product) and negative control wells were marked as + and -. The 100 bp DNA ladder (Vivantis, product No: NL1407) was marked as L. The selected positive clone shown as sharp band in 702 bp size is indicated by arrow. B. Digestion of IRES-mda-7-RGD2 with BglII restriction enzyme showed 2 fragmented patterns. The upper band is linear vector with 9594 bp size while another band (mda-7, mda-7-RGD1, or mda-7-RGD2 sequences) had 649,702, or 690 bp in length (in this image) depends on corresponding sequences. Also, 1 kb DNA ladder (Vivantis, product No: NL1411) and 100 bp DNA ladder were employed as markers. C. Digestion of plasmids with BstXI restriction enzyme: A 2-band pattern resulted after digestion with BstXI (two band of 2070 and 8020 bp for all constructs). L: The 1 kb DNA ladder, 1: undigested pIRES/mda-7-RGD2 plasmid, 2: digested pIRES/mda-7-RGD2 and 2 bands of 2070 and 8020 bp, 3: undigested pIRES/mda-7-RGD1 plasmid, and 4: digested pIRES/mda-7-RGD1 plasmid with appearance of 2 bands of 2070 and 8020 bp at the end

Immunofluorescence assay

Huh-7 cells were transfected with all plasmids for 24 hr in 8 well chamber slide (BD, USA). For staining procedure, cells were washed thoroughly with PBS before fixation in cold methanole, and then incubated at -20 ˚C. The cells were exposed to mouse anti-human IL-24 monoclonal antibody (mAb) (1:100; R&D), again washed, and then treated by Cy5 labeled goat anti-mouse IgG secondary antibody (1:500; R&D). The untransfected cells were included as negative control during staining procedures. At the end, nucleous was stained by mounting buffer containing DAPI dye to differentiate cytoplasm area and tracking viable cells. The morphology of the cells was observed immediately after staining using a fluorescence microscope (Nikon fluorescent microscopy). All the expression plasmids also contained GFP sequence, expression of which was drive by IRES sequence. Thus, GFP related green fluorescent signal play roles in assessing the rate of transfection, probable parallel expression of our genes, and evaluating the site of expressions in our experiments.

Results

Amplification of mda-7 fused with RGD motifs

Three different sequences encoding mda-7, mda-7-RGD1, and mda-7-RGD2 were amplified with our developed touch-down PCR. The amplified fragments were detected on agarose gel based on predicted sequence size. The 649 bp, 702 bp, and 690 bp fragments corresponding to mda-7, mda-7-RGD1, and mda-7-RGD2 sequences were detected in agarose 1%. The final proofed bands were extracted from agarose gel and introduced to the next step.

Cloning the genes into expression vector

After ligation of target sequences inside the TA cloning vectors, transformed colonies were selected with blue–white screening method. The suspected colonies were confirmed with colony-PCR assay which indicated that majority of clones contained desired genes. Final integrity of our genes was confirmed by bidirectional sequencing after comparing with mda-7 original gene.

The confirmed plasmids were digested with BglII and resultant fragments (mda-7, mda-7-RGD1, and mda-7-RGD2) were extracted. In the next step colony-PCR assay was performed for screening of genes inside expression vector pAdenoVator-CMV5-IRES-GFP. Then the correct plasmids were extracted from suspected clones by miniprep and introduced to orientation detecting PCR.

The positive clones were also separately digested by BglII and BstXI to evaluate the integrity. Digestion reactions made 2 fragmented patterns for BglII

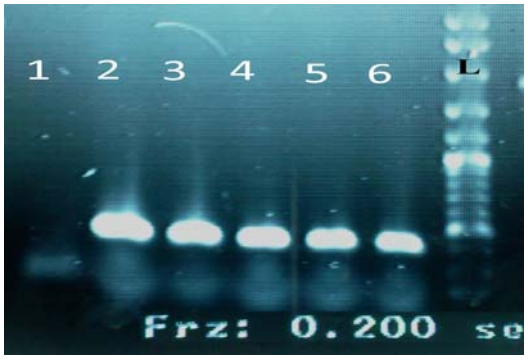

Figure 3. RT-PCR of 3 different constructs expression in HEK-293 and Huh-7 cells. RT-PCR reactions after 1% agarose gel electrophoresis. The appearance of nearly 380 bp length bands for all constructs was indicative of gene expression in both cell lines. From left to right; 1: pIRES-GFP (negative control) expression in HEK-293, 2: pIRES/mda-7-RGD2 expression in HEK-293, 3: pIRES/mda-7-RGD1 expression in HEK-293, 4: pIRES/mda-7-RGD2 expression in Huh-7, 5: pIRES/mda-7-RGD1 expression in Huh-7, and 6: pIRES/mda-7 expression in Huh-7 cells. L: 50 bp DNA ladder (CinnaGen, Cat. No: PR901633)

treatment (a linear expression vector and a band corresponding to mda-7, mda-7-RGD1 or mda-7-RGD2 sequences) and 2 fragmented patterns for BstXI treatment (near 2070 bp and 8020 bp for all construct) on agarose gel after electrophoresis, as shown in Figure 2B and 2C.

Analysis of gene expression by RT-PCR

For analysis of mda-7 gene expression total cellular RNA was extracted after transfection, then RT-PCR was performed to prepare cDNA (the expression vector with GFP gene was as negative control). The length of PCR products was predicted to be 381 bp. Analysis of agarose gel electrophoresis indicated the suitable expression of mda-7 in both HEK-293 and Huh-7 cell lines by detecting a 380 bp band as shown in Figure 3. The amount of expressed gene wasn't significantly different between traditional PCR and semi-quantification RT-PCR (unpublished data) which suggests that our modifications didn't disturb the amount of gene expression.

Direct fluorescence assay and screening for cytotoxicity

After transfection of cells by different plasmids, fluorescent screening was performed by flourescent microscopy to estimate the transfection rate. Results showed that in all treated cell groups (they received 3 test and 1 control plasmids), suitable part of cells (nearly more than 30% by eyes) were shining green by GFP expression which was indicative of successful method as depicted in Figure 4. Up to 24 hr after transfection, no significant phenotypic difference between 4 different plasmids receiving groups indicated low cytotoxic effect. This finding is concluded from appearance of cells including rounded shape, detachment, and smashed forms among GFP shining cells.

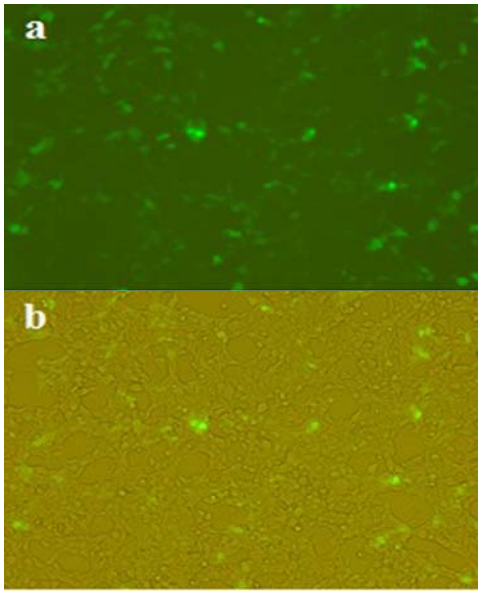

Figure 4. GFP expression in Huh-7 cells (×200). (a) Green fluorescence shining (bright green) by GFP protein under a fluorescence microscope. (b) Overlay image of light and fluorescent microscopy of the same field to estimate the rate of transfection (at least half of the cells were transfected)

Altogether, laddering test and trypan blue exclusion revealed that the relative viability and death induction by 4 different groups were not significantly different even in Huh-7 cells. While the pattern of genomic DNA in laddering experiment was similar for three mda-7 expressing and IRES-GFP control groups, the dead cell was quantified as 12%, 17%, 15%, and 20% for pIRES, pIRES/mda-7-RGD1, pIRES/mda-7-RGD2, and pIRES/mda-7-RGD, respectively. This data collectively indicated no significant survival difference between groups by our primary analysis (*P*-value >0.05). So, for further comparing of apoptosis induction between constructs, the treatment time should be extended over 24 hr and detailed assay like PI-Annexin staining method could be employed instead.

Immunofluorescence staining results

After staining the transfected Huh-7 cells with mda-7 monoclonal antibody and DAPI, by the help of natural GFP expression from constructs, informative results were obtained. Both red and green fluorescents were indicative of suitable expression of mda-7 and GFP protein, respectively; whereas DAPI blue stain showed the integrated nucleous which expected to be detected in all cells. As expected, the red signals detected inside the cells which coincidently were

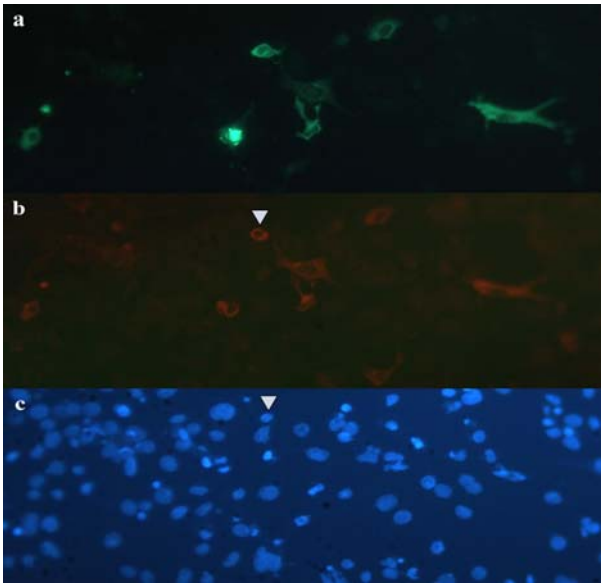

Figure 5. Immunofluorescence staining of Huh-7 transfected by pIRES-mda-7 (×400). Stained slides were evaluated by fluorescent microscopy thoroughly. Suitable signals in red, green, and blue were captured under microscope. Both green (implication for GFP) and red (indicator of mda-7 protein) were detected in corresponding cell coincidently, which confirmed the limited expression of mda-7 in transfected cells. Mda-7 expression was detected in cytoplasm but not nucleous when captured pictures were compared. DAPI blue stain showed the integrity of nucleus in majority of cells but picnotic cells demonstrated the possible cell death revealed in some transfected cells (filled triangle)

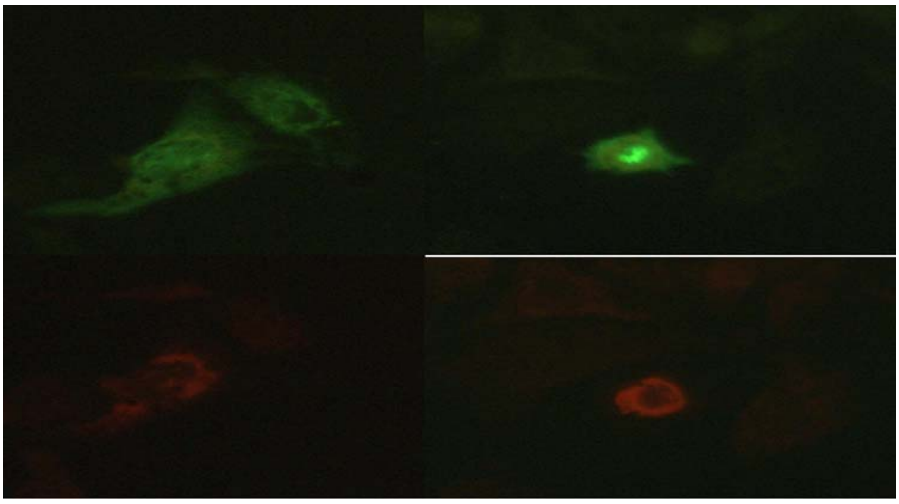

Figure 6. The closer view of IF stained slides of Huh-7 transfected cells by IRES-mda7-RGD1 plasmid (×400). More accurate image of mda-7 localization in cytoplasm after staining with specific antibody was observed under the microscopy. The localization of this protein is around the nucleous in compare with GFP protein. Unlike green GFPs (upper part of the picture) that were distributed throughout the cells, mda-7 localization (lower part of the pictures) was often limited to surrounding area of nucleous, mainly perinuclear ER region

expressing GFP protein, indicated the correlation between transfection and production of mda-7 in those cells. These results confirmed that only in transfected cells (GFP expressing cells) strong red signals were detected. Also in mentioned cells no significant cytotoxicity was detected by evaluation of nucleous shape when compared in different groups, albeit in the case of pIRES-mda-7 treated cells more picnotic cells were detected as indicative of more cytotoxicity effect (or probably cell death), as shown in Figure 5.

More importantly, these results also revealed that mda-7 protein is distributed mainly in cytoplasm but not nucleous and modification of protein sequence by RGD motif didn't change this localization pattern, as shown in Figure 5 and 6. The closer view of IF stained slides revealed that mda-7 expression is mainly limited to perinuclear ER region, as indicated before by others (26). This kind of cytoplasm localization was similar for all mda-7 modified proteins, as showed in Figure 6. This finding also supports our idea that mda-7 peptide modification will not disturb intracellular localization and consequently the way of secretion.

Discussion

Recent studies are focusing on targeted gene therapies to kill tumor cells effectively without adverse effects on other normal cells (27). Mda-7 gene is a novel tumor suppressor (2), that induces apoptosis in transformed cells without negatively affecting normal cells (28). Several studies have been performed to investigate its potential therapeutic effects and also to improve its functional properties (1, 13).

Here, we constructed three plasmids, including standard or fusion forms of mda-7 beside RGD peptides, and then their expression was examined in some human cell lines. To investigate the effect of aforementioned fusion peptides, pIRES/ mda-7, pIRES/mda-7-RGD1, and pIRES/mda-7-RGD2, constructs were prepared during some cloning steps. The expression of related proteins was evaluated via RT-PCR and IF assays. This study showed that modified mda-7 proteins were expressed in considerable rate in HEK-293 and Huh-7 cell lines. Therefore, the results suggested that these constructs are able to be employed in future researches.

To our knowledge, limited number of publications have taken advantage of RGD peptide for improving the specificity and efficacy of mda-7 in gene therapies (14, 19-20, 22). Recently Pei *et al* constructed pCDNA3.1 plasmid expressing RGD-modified mda-7(20). They created novel RGD motif in the middle of mda-7 backbone by overlapping PCR mutation, then its therapeutic efficacy was evaluated in HepG2 cell line. They demonstrated that expression pattern and apoptosis induction of new RGD-IL-24 were similar to IL-24 expression plasmid (20). Theoretically this kind of design may overwhelm true protein folding at the end or disrupt some functions, although Pei *et al* didn't mention it. Other few related experiments employed RGD targeting strategies for protein based therapy (19, 22). In other word, they added RGD peptide at the N-terminal of the protein then expressed it in bacterial host. Xiao *et al* fused RGD peptide beside mda-7 protein for the first time (19). Their group added RGD-4C to the N-terminus of IL-24 then demonstrated that both simple and fusion proteins have similar behavior for apoptosis induction and adhesion in MCF-7 cancer cell (19). Here, in order to enhance mda-7/IL-24 cell adhesion rather than elevating its inherent apoptotic inducing activity by improving its bystander effect, as a first effort,

two kinds of RGD motifs were introduced at the C-terminal of mda-7/IL-24, and then their expressions were evaluated in both normal and tumor cell lines. This kind of design would be helpful for gene based therapy and does not need protein processing in bacterial expression. This strategy will exploit both intracellular and extracellular route of apoptosis through endogenous expression and subsequent secretion into surrounding cells, respectively.

Of 25 known integrins, eight types bind to RGD (Arg-Gly-Asp) peptide which serves as the main integrin recognition site in the extracellular matrix proteins (29). Hence fusing RGD peptides is supposed to augment tumor cell targeting and show higher apoptotic rate in transfected cells through a positive feedback loop. Integrin α5β3 has been shown to be expressed on a variety of hepatocellular lines like Hep-G2, but only in negligible level in Huh-7 line (30). So, to explore intracellular expression and avoid extracellular effect of mda-7 low α5β3 integrin cell, Huh-7 was used in our project.

Our selected RGD motifs had fine differences in sequence as original cyclic RGD-4C (A**C**D**CRGDC**F**C**G); harbouring at least 2 more amino-acids at terminal compared to the smaller version. Theoretically, original size of mda-7 may exhibit immune induction, which will be avoided in our smaller RGD2 version (**C**D**CDGRC**F**C**). This kind of design will avoid immune induction, albeit keeps the targeting potency of modified RGD peptide intact, as reported before (21).

The laddering test and trypan blue exclusion revealed that viability of 3 different cell groups receiving test plasmids was not different significantly, over 24 hr after transfection. It is demonstrated that mda-7 plasmid without RGD peptides induces more cytotoxicity (but not at significant level) in compare to other groups. Thus more detailed analysis of apoptosis induction should be performed to figure out the exact difference between all mda-7 constructs. These results also confirmed that in transfected cells (GFP shining cells) strong red signals can be detected, but in those cells no significant cytotoxicity is detected. In cell group that received mda-7 plasmid, more picnotic cells were observed under fluorescence microscope which indicated more cytotoxicity effect and probably more death, as shown in Figure 5.

More importantly, our results revealed that mda-7 was distributed mainly in the cytoplasm and its modification by RGD motif didn't change the localization pattern around perinuclear ER compartments, as described before (26). This finding emphasizes that our 2 new plasmids expressing mda-7 beside RGD motif, produce enough amount of protein inside HUK-293 and Huh-7 cells and hence localization pattern is similar for all proteins.

Conclusion

Whereas our constructs integrity and expression potency were validated, to compare detailed apoptosis between these constructs, treatment time should be extended over 24 hr, more sensitive assay like PI-Annexin staining should be employed, and Huh-7 should be replaced by high integrin expressing cell like Hep-G2 to evaluate precise bystander effect. Mda-7 targeting by RGD peptide seems more appealing than applying mda-7 alone, thus it is valuable to assess type and site of RGD peptide modification then evaluate its potency *in vitro* and *in vivo* for gene therapy approaches.

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