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Postal Code: 9177948953
P.O. Box: 917751436
Tel: +98-513-8804063
Fax: +98-513-8795162
E-mail: jcmr@um.ac.ir
Online Submission: http://jcmr.fum.ac

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Research Article

Comparison of Human Factor IX Abundance and mRNA Expression Levels In Stable Insect and Mammalian Cells

Jafar Vatandoost[1]*, Shadi Tirdad[2]

[1] *Department of Biology, Hakim Sabzevari University, Sabzevar, Iran*
[2] *Department of Biotechnology, Islamic Azad University, Sabzevar, Iran*

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Abstract

In the production of recombinant proteins, the selection of an expression system is very important. Although CHO cells are used as mammalian expression system, the ability of insect Schneider line 2 (S2) cells as a new expression system for the high production of many human proteins were confirmed. It is suggested that high copy number of an introduced gene and so high transcription in these cells can be regarded as one of the reasons for high expression of recombinant proteins. Therefore, the present study aimed to evaluate the correlation of recombinant human coagulation factor IX (hFIX) abundance and mRNA expression. The amount of hFIX mRNA by quantitative real-time PCR as well as hFIX protein in cell lysate and cultured media by Elisa was analyzed. The results of data analysis indicated 6 fold increases in mRNA level in S2 cells in comparison to CHO cells. Furthermore, S2 cell line indicated 5.5 and 7-fold increase in total and secreted protein level, respectively, compared to CHO cell line. The data demonstrated the correlation of mRNA and protein abundance and indicate that S2 cell lines are superior in producing the recombinant proteins.

Keywords: Drosophila S2 cells, CHO cells, Coagulation factor IX, Real-time PCR, Elisa

Introduction

Gene expression is a process by which information from a gene is used in the synthesis of a functional gene product. Several steps in the gene expression process may be modulated such as DNA-RNA transcription step to post-translational modification of a protein (Myhre et al., 2013). Moreover, in the production of recombinant proteins, expression of a foreign gene is influenced by several factors such as copy number of introduced gene, and the ability of cell line in transcription and translation (Brown, 2016; Wikibooks, 2017). Therefore, using an expression system is important in the regulation of gene expression including a wide range of mechanisms to increase the production of specific gene products.

Among the various protein expression systems, a cell system used increasingly for the expression of various recombinant proteins is based on *Drosophila melanogaster* Schneider line 2 (S2) cells, which offers a straightforward approach to stably produce high quantities of a functional protein (Moraes et al., 2012). The S2 cells were developed as a plasmid-based and non-lytic integration system, capable of stably expressing high levels of recombinant proteins (Bernard et al., 1994; Kirkpatrick and Shatzman, 1999). The S2 expression system has the advantage of proliferating without requiring CO2, being easily adapted to large-scale fermenters, being capable of long-term continuous culture, having a null expression background, and having the appropriate post-translational machinery (Moraes et al., 2012; Vatandoost et al., 2012). Correct post-translational modification of a recombinant protein especially in the case of hFIX influence on production of protein and so indicated as an important factor in the selection of an appropriate host cell. Moreover, the Drosophila metallothionein (Mtn) promoter is shown to be tightly regulated and capable of driving high-level heterologous transcription upon induction by metals such as Cu or Cd ions (Maroni et al., 1986; Otto et al., 1987). Furthermore, Drosophila cells can integrate up to 1000 copies of an expression cassette in a single transfection event while protein expression remains tightly regulated even at high copy numbers with low basal expression, maintained in the absence of an inducer (Kim et al., 2008). By considering these advantages, it seems that insect S2 expression

Corresponding authors E-mail:
**j.vatan@hsu.ac.ir*

system has the mechanisms to increase the production of recombinant proteins. Using this system, a wide variety of proteins that are appropriately processed and are able to maintain biological activity have successfully been expressed in large scale (Moraes et al., 2012). The reason for high expression is still in question in spite of demonstrating a high expression of recombinant proteins in S2 cells. It is believed that high copy number of introduced gene and so high mRNA abundance can play a significant role and is correlated with the expression of recombinant proteins. By considering all this issues, the expression of hFIX as a recombinant protein at the mRNA and protein level was evaluated in the present study. In other hand, a new expression system, *Drosophila melanogaster Schneider* line 2 (S2) cells, was taken into consideration in comparison with mammalian CHO cells.

Materials and Methods

All the enzymes used for the molecular biology, the kits for PCR purification and plasmid isolation, RNA preparation, reverse transcriptase (M-MuL V), and other chemicals such as protease inhibitors were purchased from SinaClon in Tehran, Iran. Oligonucleotides were synthesized by Denazist (Mashhad, Iran) and Bioneer (Daejeon, Republic of Korea). The pcDNA3, pMT-V5-HisA and pCoHygro plasmids and all cell culture reagents were got from Thermo Fisher Scientific, except Schneider's insect medium, penicillin G and streptomycin (Sigma-Aldrich). Further, Geneticin (G418), hygromycin, vitamin K and protease inhibitors were purchased from Roche and the enzyme-linked immunosorbent assay (ELISA) specific for human FIX (AsserachromhFIX:Ag, Stago, France) and activated partial thromboplastin time (aPTT) reagents were purchased from Diagnostica Stago (Bern, Switzerland).

Cell culture and Preparation of Stable Clones

Drosophila Schneider (S2) and CHO cells as a kind gift from Dr. A.R. Zomorodipour, NIGEB, Iran were cultured and transfected as described previously (Haddad-Mashadrizeh et al., 2009; Vatandoost et al., 2012). In summary, the CHO cells were grown in a 5% CO_2 atmosphere at 37°C, sub-cultured at a density of 2×10^5 cells in a volume of 2 ml in 6-well plates, and were transfected with 2 µg pcDNA3-hFIX by using FuGene–6. Individual clones were expanded in the presence of 450 µg/ml geneticin. Expression media containing 6 µg/ml of vitamin K1 was added to ~70% confluent cells, upon which the individual clones were screened for FIX production. S2 cells were kept at 28ºC without CO2 under the normal atmosphere in Schneider's insect medium, supplemented with penicillin G (50 units/mL) and streptomycin (50 µg/mL). One day before transfection, 3×10^6 cells were seeded in a volume of 3 ml in 6-well plates, upon which the cells were allowed to loosely adhere. The S2 cells were transfected with pMT-hFIX and the pCoHygro plasmid, including the hygromycin resistance gene by employing the calcium phosphate co-precipitation method with minor modifications (3). After 48 hours, individual clones were selected in the presence of 300 µg/ml hygromycin B. The clones were screened for FIX expression, followed by the induction with 0.5 mM CuSO4 in the presence of 6 µg/ml vitamin K1.

Analysis of hFIX Transcript Levels with Quantitative Real-time PCR.

The total RNA was prepared from the induced cells by using RNX-Plus Kit (SinaClon, Iran), based on the manufacturer's instructions and was reversely transcribed by using random primers and revertAid M-MuLV. The cDNA synthesis was done by using specific primers, hFIX-F (5'GAATGTTGGTGTCCCTTTGG3') and hFIX-R (5'AATGGCACTGCTGGTTCAC3'). Using a total of 100 ng of RNA from each cell line, real- time PCR was performed, using SYBR green method and premix Amplicon kit on an ABI 7500 real-time PCR system. The ribosomal protein L32 (RPL 32) for S2 cells and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) for CHO cells, were considered as the internal control. A 165 bp and 123 bp fragments of RPL 32 and GAPDH were amplified through using oligonucleotides RPL-F (5' ATCGGTTACGGATCGAACAA 3')/RPL-R (5' GACAATCTCCTTGCGCTTCT3') and GAPDH-F (5'-AGGTCGGTGTGAACGGATTTG-3)/ GAPDH-R (5'-TGTAGACCATGTAGTGGTCA-3'), respectively. The real-time PCR conditions consisted of a pre-denaturation step at 95°C for 5min, followed by 40 cycles at 95°C for 15s and 58°C for the 20s.

Quantification and Activity Analysis of Recombinant hFIX by ELISA and aPTT

Human FIX was quantified in conditioned media by employing an ELISA based on the procedure provided by the manufacturer. In the procedure, strip wells were pre-coated with goat polyclonal antibody to hFIX.

Then, the samples were diluted and 100 µl of text sample were applied to the wells. After 30 minutes of incubation to the present hFIX, antigen binds to

the coated antibody, unbound material washing away and, 100 µl peroxidase-labeled FIX detecting antibody were applied and allowed to bind to the captured hFIX for 30 minutes. Then, the wells were rewashed and a solution of TMB (100 µl of peroxidase substrate tetramethylbenzidine) was applied and allowed to react for 10 minutes. A blue color is developed and changed to yellow upon quenching the reaction with 100 µl of 0.2 M sulphuric acid.

Finally, the color formed is measured spectrophotometrically in a microplate reader at 450 nm. The absorbance at 450 nm is directly proportional to the concentration of hFIX, based on the standard curve as stated in ng/mL. The assay is calibrated by using the calibrator plasma provided in the kit. Moreover, the results obtained after the subtraction of non-specific absorbance were determined on cultured media from the non-transfected cells. In addition, intracellular accumulated hFIX was assessed, for which the cells were pelleted by centrifugation at 100 g for 5 min, upon which they were resuspended in 500 µL of ice-cold lysis buffer (100 mM KCl, 2 mM MgCl2, 10 mM, HEPES pH 7.5, 0.5% Triton X100), including an antiprotease mixture combined of completed Protease Inhibitor (Roche). Subsequently, the lysate was centrifuged at 12,000 g for 10 min at 4 ºC and the supernatant was assessed for hFIX.

The functional activity of recombinant hFIX was examined using an aPTT assay (Otto et al., 1987). First, human plasma immuno-depleted of FIX (100 µL) was mixed with conditioned media (100 µL) and aPTT reagent (100 µL). After 3 min of incubation at 37 ºC, 100 µL of a prewarmed CaCl2 solution (25 mM) was added to the mixture, and the clotting time was recorded. Then, the activity of the expressed hFIX was calculated based on the standard curve of normal human plasma (Iranian blood transfusion organization), and one unit of FIX activity corresponded to the amount of FIX in 1 ml of normal plasma (~ 5 µg/ml).

Data Analysis

All expression analysis of the experiments was carried out in duplicates or triplets, and the generated data were presented as the mean ± SD. ANOVA was used for data analysis, followed by a Tukey post-hoc test. $P<0.05$ was considered as the level of significance.

Results

In this study, mRNA level and expression of the hFIX in stable hFIX producing CHO and S2 cells were investigated. After induction, the presence of FIX mRNA in both cell types was confirmed by employing RT-PCR. Following determining of quantity, purity and integrity of extracted RNA using spectrophotometry and electrophoresis, PCR for hFIX, GAPDH and RPL32 genes was performed. Observation of single bands for hFIX, RPL32 and GAPDH gene which was 182 bp, 165 bp and 123 bp, respectively, indicated the accuracy of amplification of the target gene (Fig. 1)

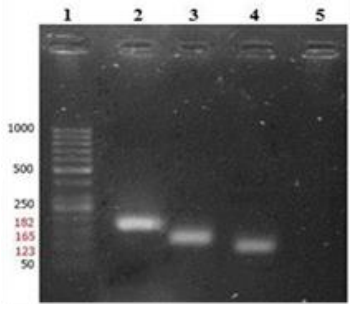

Figure 1. Gel electrophoresis of RT-PCR products of hFIX, RPL32 and GAPDH. Electrophoresis of PCR products obtained by RT-PCR on 2% agarose gel electrophoresis. Lane 1, 50 bp DNA ladder (Thermo Scientific); lanes 2, 3 and 4, the amplification product of hFIX (182 bp), RPL32 (165 bp) and GAPDH (123 bp); lane 5, negative control.

Real-time PCR analysis showed that both of cell types were able to express hFIX effectively, and the hFIX transcript level in S2 cells was higher than that of the CHO cells (Fig. 2).

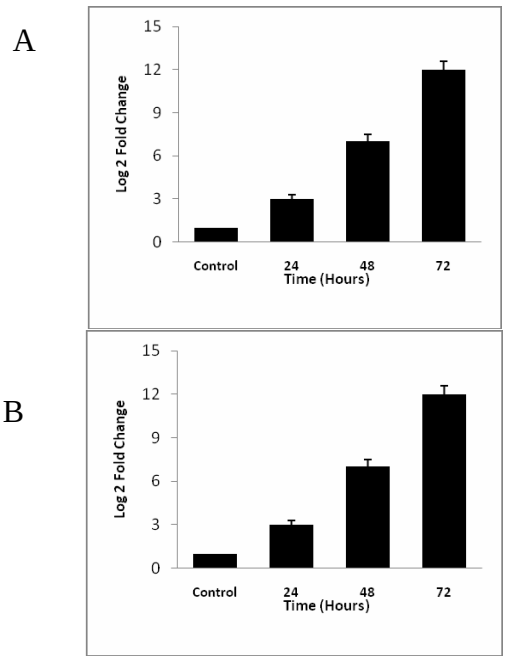

Figure 2. Real-time PCR analyses for the assessment of hFIX. The hFIX mRNA levels in stable S2 (A) and CHO (B) cells during 72h after induction assessed by real-time RT-PCR. Data are mean ± SEM

Research Article

Compared to non-transgenic cells, there was a significant increase in gene expression during 72 hours in both cells (P <0.05). Further, RNA expression levels were reached to 12 times after 72 hours in comparison to the control gene, which was 2 fold in case of CHO cells. Moreover, the comparison of RNA expression levels showed 4.5 fold increases in S2 cells than CHO cells. To evaluate the hFIX expression in hFIX producing stable CHO and S2 cells, cell lysate and conditioned media were obtained and subjected to ELISA to measure the hFIX concentration. Detectable hFIX levels were achieved in both cells although there was a significant difference in hFIX levels between the hFIX producing stable S2 and the CHO cells (P>0.05). The highest total hFIX content in 72h was 502 and 91 ng/ml/10^6 cells in S2 and CHO cells, respectively, through which, 432 and 63 ng/ml/10^6 cells were secreted to media (Fig 3). As the secretion efficiency of a particular protein is defined as the ratio between the secreted fraction and its total amount, the ratio of secreted hFIX to total hFIX protein was calculated. The highest secretion efficiency of S2 cells was 86%, in comparison to 68% in CHO cells.

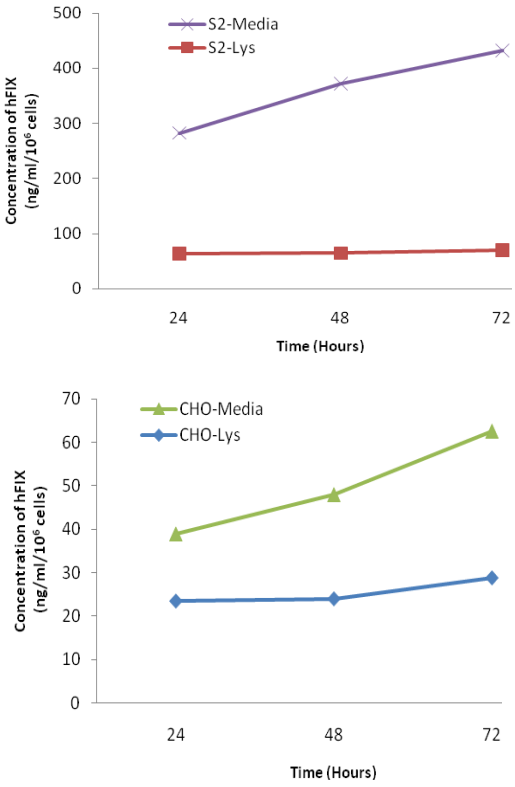

Figure 3. Evaluation of the hFIX expression in stable CHO- hFIX and S2- hFIX cells. hFIX levels in stable CHO (B) and S2 (A) cells were determined by Elisa at various post-induction times. ELISA performed on samples taken from both the culture supernatant and the cellular fraction. Data are mean ± SEM

Discussion

Optimizing the generation of the recombinant proteins in laboratory expression systems is important for the purpose of the reduction of the production cost since the treatment of many of diseases including Hemophilia-B is done by replacement therapy of recombinant proteins. There are many ways to increase expression and the activity of the recombinant proteins in expression systems (Vatandoost and Pakdaman, 2016) such as the expression systems with the capability of more active protein production. In recent years, the S2 cells with various advantages including high growth rate have been introduced as an efficient expression system. Because of the capability of these cells to integrate multiple copies of expression plasmids in a transfection event (Cherbas and Cherbas, 2007; Moraes et al., 2012), it was expected that the number of the transcribed mRNAs would be high. The comparison of the expression of coagulation factor IX in S2 and CHO cells showed that the amount of the expression of FIX mRNA in S2 cells is about 6 times higher than CHO cells. Regarding the reasons, we can refer to the high copy number of the integrated plasmid in S2 cells in comparison with CHO cells, and the power of the used promoter in vectors. It seems that the Mtn promoter is more powerful although the CMV promoter in mammalian vectors is a constitutive powerful promoter. Further, it is tightly regulated (Johansen et al., 1989) and this is, especially crucial when expressing proteins that may have a metabolic board to growing cells.

Protein levels are influenced by the regulation of transcription, translation and protein stability (Myhre et al., 2013). Based on FIX mRNA level in S2 and CHO cells, it is also expected that the amount of produced protein will be about 6 times. However, the results of the total amount of protein in two cells indicated that this ratio is about 5.5 times. In consistent with the previous results (Myhre et al., 2013), the results of the present study indicated that mRNA expression was correlated significantly to protein abundance. Therefore, the more mRNA in S2 cells results in more protein production although there is another mechanism like translation and translocation to ER that may be effective in this process. In addition, the amount of secreted proteins to cultured media is important since the purification simplicity of the recombinant proteins is a significant factor in their production process. The assessment of secreted FIX to cultured media indicated that while FIX was secreted at levels up to 63 ng/ml/10^6 on day 3 post-induction in CHO cells,

the highest secreted FIX in S2 cells reached to 432 ng/ml/10^6 that was seven-fold more. Furthermore, although high densities of S2 cells rather than CHO cells can have an influence on the amount of FIX in cultured medium, in comparison to one million cells, they showed that high expression in S2 cells might be affected by factors such as the transcribed mRNA level, translation and translocation capability. It was expected that higher mRNA and so higher produced FIX might result in more secretion. However, the results indicated that by increasing FIX expression, the secretion efficiency of FIX in S2 cells increased during 24 to 72 hours. Furthermore, the secretion efficiency of FIX in S2 cells with more protein expression was about 15% higher than CHO cells.

In conclusion, the S2 cells were superior to mammalian cells in expression of recombinant proteins from mRNA to protein level.

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Research Article

Differential Expression of EGFR, MAP2K4 and E2F3 Genes as Targets of miR-141 and Its Association with Immune System Pathway

Soheila Shokrollahzade[1], Shamim Sarhadi[2], Majid Safa[3,4], Arshad Hosseini[1,3*]

[1] *Department of Medical Biotechnology, School of Allied Medicine, Iran University of Medical Sciences, Tehran, Iran*
[2] *Department of Medical Biotechnology, Faculty of Advanced Medical Sciences, Tabriz University of Medical Sciences, Tabriz, Iran*
[3] *Cellular and Molecular Research Center, Iran University of Medical Sciences, Tehran, Iran*
[4] *Department of Hematology, School of Allied Medicine, Iran University of Medical Sciences, Tehran, Iran*

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Abstract

MicroRNAs by their structural complementarity capabilities have canonical roles in gene regulation. In this paper; we investigate expression of EGFR, MAP2K4 and E2F3 genes targeted by miR-141, a member of miR-200 family. EGFR, MAP2K4 and E2F3 were predicted as the potential targets of mir-141 by using online miRNA bioinformatics tools. MCF-7 cells were transfected with mir-141-precursor and inhibitor vectors. Expression of miR-141 and target genes was determined by using qRT-PCR. To see the most relevant pathways regulated by miR-141, we constructed two separate networks by NetworkAnalyst and enriched list of underlying genes by Enrichment analysis tools. The expression changes of all three predicted targets were higher in transfected cells with anti-mir-141 vector, compared with the control untransfected cells. By contrast, in transfected cells with pre-mir 141, we did not see significant expression changes in EGFR, E2F3 and MAP2K4. List of genes in total networks as well as explored functional modules were enriched separately. Enrichment analysis shows that immune system pathway has the strongest relationship with the proteins potentially targeted by miR-141. The present study demonstrated potential role of miR-141 in regulation of EGFR, MAP2K4 and E2F3 expression and suggested innate immunity pathways as the key pathway through which this regulatory network contributes to breast cancer development.

***Keywords*:** Breast cancer, MiR-141, EGFR, MAP2K4, E2F3

Introduction

Ranked as the second leading cause of cancer death worldwide, breast cancer is a major health concern. The current trend underlines the need for a greater understanding of the molecular biology of breast cancer. With regard to breast cancer development, microRNA regulation of cancer-related pathways plays an important role.

MicroRNAs are short non-coding RNAs with 16-22 nucleotides. MiRNA seeds can make imperfect matches either on 3'-UTR or 5' UTR of targeted mRNAs.

Following this imperfect match, numerous biological events can occur, including translational inhibition, mRNA complete and incomplete degradation (Xu et al., 2013) . Since miRNAs regulatory mechanisms through seed matching endowed it with a master regulatory role, the targets could be as numerous as possible. miRNAs control many biological processes and molecular functions including; tumorigenesis, metastasis, drug resistance, invasion, self-renewal and proliferation.

Since the first reports that demonstrated relationship of pathological Epithelial to Mesenchymal Transition and cancer stem cell with miR-200 family in breast cancer, many researchers choose members of miR-200 family as a vital potential candidate in cancer (Park et al., 2008; Shimono et al., 2009). miR-200 family includes five members, miR 200a/b/c, miR 429 and miR-141. miR 200a/b and miR 429 located on human chromosome 1, miR-200c and miR-141 located on human chromosome 12. MiR-141 is short structured with low GC content(Kim et al., 2012) . Many studies showed that members of miR-200 family act as a tumor suppressor in cancer.

To address some of those important studies, cell growth, cell proliferation and metastasis in tissue samples and cell lines of hepatocellular carcinoma have been shown to be strongly regulated by miR-141 (Xue et al., 2014). Analysis of miRNA-mRNA interactions in MDA-MB-231 cells revealed down regulation of miR-141 in invasive cell lines (Luo et

Corresponding authors E-mail:
**hoseini.a@iums.ac.ir*

al., 2013). In addition, bioinformatics analysis in miRNA profiling of bladder cancer patients indicated miR-141 as a hub-miRNA and E2F3 as a hub target gene (Canturk et al., 2014)

To investigate targets of miR-141 in MCF-7 breast cancer cell line, we applied online databases and software. By looking in overlapping results from different software, we predicted E2F3, MAP2K4 and EGFR as the potential targets of miR-141.

E2F3, among other members, causes transition of cells from G1 to S phase by interacting with pocket protein Retinoblastoma. Through this interaction, Rb is phosphorylated and inactivated, resulting in release of E2F3 molecule. E2F3 employs cycline E and to initiates transcription of cascades of genes (Shimono et al., 2009). E2F3 causes the transition of cells from G1 to S phase by employing cycline E and CDK2. Transcription of E2F3 initiates transcription of cascades of genes, leading to cell cycle progression. In breast cancer, studies in four different cell lines have shown that responsible receptors for proliferation pathways is highly regulated by E2F3, especially in ER positive cell lines (Nguyen-Vu et al., 2013). MAP2K4 belongs to the protein kinase superfamily. Activation of protein kinase MAP2K4 initiates phosphorylation of p38 and JNK that results in phosphorylation of Stress Activated Protein Kinase (SAPK) pathway (Marasa et al., 2009). To date, many discrepancies have been found about role of MAP2K4 as a tumor suppressor or pro-oncogene in cancer. Even in a same type cancer, many controversies are raised about this major signaling molecule. In some studies, expression of MAP2K4 is increased by downregulation of mir-141(Marasa et al., 2009).

EGFR ligand binding switches on a cascade of downstream signaling pathways including RAS-RAF-MEK-ERK, PI3 kinase-AKT, PLC gamma-PKC, STATs. Increased expression of EGFR has been proved to result in Erk-induced EMT, increased tumor size and chemotherapy resistance in triple Negative Breast Cancer (TNBC) and Inflammatory Breast Cancer (IBC) (Masuda et al., 2012).

Strong evidence of correlation of EGFR and miR-200 family member are provided by Uhlmann et.al in 2010. MiR-200bc/429 and miR-200a/141 regulate cell cycle progression through p27/Kip1 (Uhlmann et al., 2010).

In this study, expression changes of E2F3, MAP2K4 and EGFR following ectopic transfection of pre-mir-141 and anti-mir-141 is investigated. To suggest the most probable associated signal pathways with mir-141- target genes circuit, two different PPI (Protein-Protein Interaction) network regulated with miR-141 is constructed and functional modules of each PPI networks are detected. PPI networks are then enriched, using Enrichr.

Materials and Methods

Cell Line and Culture Condition

MCF-7 cells (human breast adenocarcinoma cell line) were purchased from Iranian Biological Resource Center (IBRC). MCF-7 cells have been cultured in DMEM/F12 medium (Caisson Labs, USA) supplemented with 10% Fetal Bovin Serum (Atocel, Austria), with 1% Penicillin/Streptomycin (Biowest, Canada) antibiotic. Cells were grown in 5% CO2 at 37-degree incubator, humidified 90%.

Construction of MiR-141 Precursor (Pre-miR-141) and MiR-141 Inhibitor (Anti-MiR-141) Vector

Genomic sequence of mir-141 precursor, extracted from human normal White Blood Cells was amplified by Pfu polymerase (GeneAll, South Korea), using CCCTGTAGCAACTGGTGAGC as forward primer and CCCTGAAGGTTACTGCCGAG as reverse primer. The PCR product is then ligated into pCR®2.1 (Invitrogen, USA) and transformed into DH5α competent cells (TaKaRa, Japan). Ligated sequence was directly cloned into pTracer™-SV40 vector (Invitrogen, USA) and transformed to DH5α competent cells (TaKaRa, Japan). Zeocin™ antibiotic (Life Technologies, USA) was used as the selective marker. mir-141- pTracer™-SV40 vector was further purified by Plasmid Midiprep Kit (Qiagene, USA) according to the protocol.

PLenti-III-mir141-off vector and mock vector were chemically synthesized by ABM (Canada) and then transformed into DH5α competent cells (TaKaRa, Japan). Positive selected clones were chosen and purified by Plasmid Midiprep Kit (Qiagene, USA) according to the protocol.

Transient Transfection

MCF-7 cells were transfected with mir-141-pTracer™-SV40 vector and pLenti-III-mir141-off vector in 2 different 24-well plates. A day prior to transfection, 110000 cells were seeded into a 24-well plate. Cells were seeded into plates with complete DMEM/F12 medium supplemented with 10% FBS cells and were kept in antibiotic free media. Cells were transfected with P-tracer SV40- mir141, PLenti-III-mir141-off vector and mock vector as well. Fluorescent microscope (Olympus IX2-RFACA) was used to monitor the transfected cells

for GFP-positive signals 24h and 48h after transfection.

MTT Assay

Cell viability was determined 48 hours' post-transfection by MTT method. 20 * 10^4 cells were seeded in 96-well plates and cultured for 24 hours before transfection. Cells were then transfected with 1µg of pre-mir 141 as well as anti-mir 141 vectors for 48 hours. 1X MTT working solution (Atocel, Austria) was prepared using Phosphate Buffer Saline. 10µl of 1X MTT working solution was added to each well and incubated for 4 hours in co2 incubator 100 µl of DMSO was added as solubilization buffer. Absorbance was measured at 590 nm using a microtiter plate reader (BioRad, USA). Viability of untreated cells was set at 100%, and absorbance of wells with medium and without cells was set as zero.

RNA Isolation, Reverse-Transcription and Real-Time qRT-PCR for Mir-141 and Target Genes

Total RNA was extracted by Tripure (Roche Applied Science, Germany), then was used as the template for Reverse transcriptase reaction. Reverse transcription was performed using stem-loop RT primers providing in the kit (Biorbyte, England). Real-time quantitative PCR analysis was performed using hsa-miR-141 Real-time Detection kit and U6 Calibration (Biorbyte, England). RT Random hexamer primers were used for revers transcription according to the manufacturer (Roche Applied Science, Germany). Real-time quantitative PCR analysis was performed using QuantiTect SYBR® Green PCR Kits (Qiagene, USA). Primer Sequences of predicted target genes are given (Table 1).

Table 1. Primer sequences of EGFR, E2F3 and MAP2K4 genes

1- EGFR	*Forward primer* *5'* TGCCACCTGCGTGAAGAAG *3'*
	Reverse primer *5'* ACCTATTCCGTTACACACTTTGC *3'*
2- E2F3	*Forward primer* *5'*GCCTGACTCAATAGAGAGCCTAC *3'*
	Reverse primer *5'* AGTCTTTGGAAGCGGGTTTAGG *3*'
3- MAP2K4	*Forward primer* *5'* ACTTCGGCATCAGTGGACAG *3'*
	Reverse primer *5'* GACATCAGAGCGGACATCATATC *3'*

Bioinformatics and Statistical Analysis:

Target Prediction

Online bioinformatics tools are applied to predict targets of miRNA-141. Targets with the highest score matching to miR-141 which were also overlapping in Target Scan, Mir Walk, mirBASE, mir Map and miRANDA were predicted.

PPI Network Construction

Two discrete PPI networks are differently seeded and constructed. For the first network, that is Network A, EGFR, MAP2K4 and E2F3 proteins are considered as seeds. The second PPI network, Network B, is seeded by target proteins predicted by miRmap. 60 proteins with the probability of >99% to be targeted by miR-141 is retrieved from miRmap. To find the corresponding proteins, target genes were mapped on PPI network which is used by NetworkAnalyst, including high-quality protein-protein interaction (PPI) database based on InnateDB that also cover other PPI databases like IntAct, MINT, DIP, BIND, and BioGRID. Of these targets, 51 proteins are selected as seeds of the network. Networkanalyst is used for network construction and visualization.

Functional Module Analysis

In the next step, NetworkAnalyst module detector is employed to find functional modules of these two constructed networks. Top three functional modules of each network are screened and Functional Pathway Enrichment Analysis is done by Enrichr. Pathways are then studied using Reactome 2016.

Results

Target Prediction

Table 2 to 4 show predicted consequential pairing of target region and miRNA. The type of seed matching is also indicated. Position of the predicted target genes that will be complemented to has-miR-141-3p or has-miR-141-5p has been indicated in the first right column in table 2.

Seed match sequence is also represented graphically in the second column. Description of types of seed matching that is shown in the last columns. Table 3 shows possible complementarity of has-miR-141-3p or has-miR-141-5p with EGFR, MAP2K4 and E2F3 based on miRmap online target prediction tool. Structural properties as well as miRmap score are also represented. Table 4 shows miRDB results of predicted targets for has-miR-141-3p. Location of seed sequences, target ranks and scores are shown.

Table 2. List of predicted targets by Target scan showing the type of seed matching

	Predicted Consequential Pairing of Target Region (top) and miRNA (bottom)	Seed Match
Position 69-75 of E2F3 3' UTR hsa-miR-141	5' …AGAACAUCUGUCAUGCAGUGUUG… ||||||| 3' GGUAGAAAUGGUCUGUCACAAU	7mer-m8
Position 2603-2610 of E2F3 3' UTR hsa-miR-141	5' …ACAUGAGCUGUCAAACAGUGUUA… ||||||| 3' GGUAGAAAUGGUCUGUCACAAU	8mer
Position 2771-2777 of E2F3 3' UTR hsa-miR-141	5' ...UUGUAAUUUUUUAAGAGUGUUAU... |||||| 3' GGUAGAAAUGGUCUGUCACAAU	7mer-1A
Position 28-34 of EGFR 3' UTR hsa-miR-141-3p	5' …AUAUAAAUGGGAAAUCAGUGUUU… ||||||| 3' GGUAGAAAUGGUCUGUCACAAU	7mer-m8
Position 75-81 of MAP2K4 3' UTR hsa-miR-141	5' …UUUCAUCCCGUAUCACAGUGUUU… ||||||| 3' GGUAGAAAUGGUCUGUCACAAU	7mer-m8
Position 192-199 of MAP2K4 3' UTR hsa-miR-141	5' …ACCUGAUUGAUCACACAGUGUUA… ||||||| 3' GGUAGAAAUGGUCUGUCACAAU	8mer

* 7mer-m8: An exact match to positions 2-8 of the mature miRNA (the seed + position 8)
** 8 mer: An exact match to positions 2-8 of the mature miRNA (the seed + position 8) followed by an 'A'

Table 3. List of predicted targets by miRmap

miRNA	**Gene**	**ΔG Open**	**Probability Exact**	**miRmap Score**
hsa-miR-141-5p	EGFR	95.42	95.13	98.54
hsa-miR-141-3p	MAP2K4	76.45	97.05	98.29
hsa-miR-141-3p	E2F3	77.88	77.15	97.55
hsa-miR-141-3p	EGFR	71.78	66.87	87.11
hsa-miR-141-5p	E2F3	55.93	48.53	49.72

Table 4. List of predicted targets by miRDB

MiRNA Name	**Gene Symbol**	**Target Rank**	**Target Score**	**Seed Location**	**3'UTR Length**
hsa-miR-141-3p	EGFR	10	81	1488	1737
hsa-miR-141-3p	E2F3	3	68	69, 2603	3302
hsa-miR-141-3p	MAP2K4	23	77	75, 192	2571

Viability of MCF 7 Cells

In this study, MTT assay was done to ascertain whether viability of MCF-7 cells has declined after transfection and the transfection reagent caused any toxicity to cells. As seen in figure 1, viability of cells transfected with mir-141 precursor and inhibitory vector compared to control untransfected cells, has shown 0.02 and 0.09 differences respectfully. Since this is not a significant change, we conclude that transfection process itself, have not changed viability of MCF-7 cells.

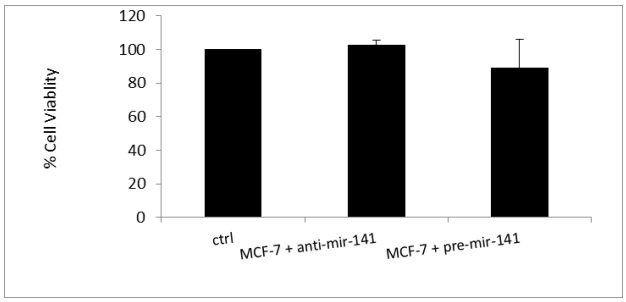


Figure 1. Cell viability (MTT assay) 48 hours after transfection.

Figure 1 shows MTT assay in 3 different groups of cells 48 hour after transfection. No significant changes in viability of cells are seen after transfection.

GFP Expression and Death of Transfected Cells

GFP expression was confirmed by fluorescent microscopy 48 hour following ectopic expression of mir-141 inhibitory as well as overexpression vector. All transfected cells with mock vector were also checked to make sure that cell death is not resulted from plasmid transfection (figure 2).

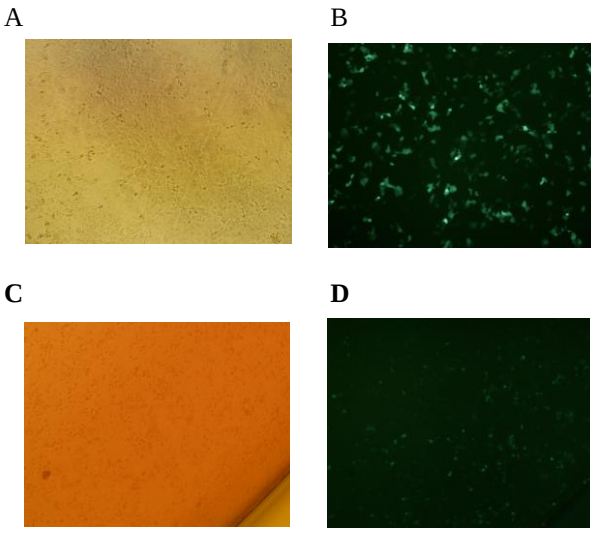


Figure 2. GFP signal of MCF-7 cells 48 h after transfecting by mir-141-Tracer and inhibitory vector. a) Optical microscopy of transfected MCF-7 cells with anti-mir-141 b) Fluorescent microscopy of MCF-7 transfected with anti-mir-141 vector after 48-hour for the same slide, c) Optical microscopy of transfected MCF-7 cells with pre-mir-141 vector, d) Fluorescent microscopy of MCF-7 transfected with pre-mir-141 vector.

Expression Level of MiR-141 in MCF-7 Cells

Decreased expression level of miR-141 was determined in cells transfected with mir-141 inhibitory vector, using real-time PCR. Data was normalized against U6 RNA. Using student t-test, the discrepancy between these groups was statistically significant ($p < 0.05$). By contrast, in cells transfected with pre-mir 141, increased expression of miR-141 is not statistically significant (figure 3).

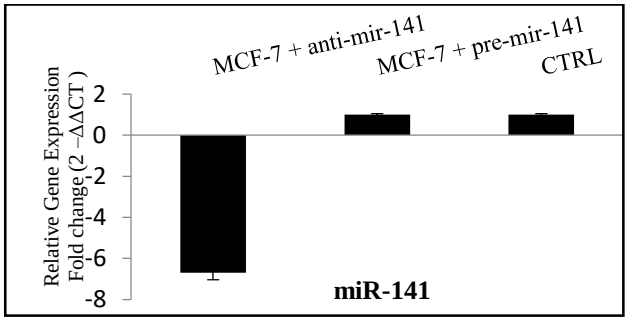


Figure 3. Expression level of miR-141 in transfected cells by real time-PCR.

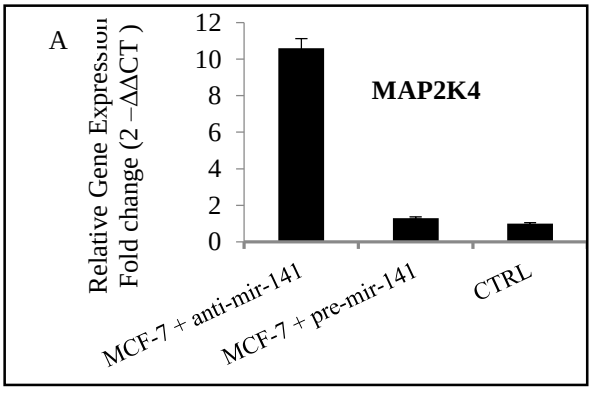
Figure 3 shows the relative expression changes of miR-141 in untreated cells as control group as well as transfected cells with pre-mir-141 and anti-mir-141 vector. Data is normalized with U6 as housekeeping gene. For relative quantification of gene expression, real time-PCR test has been repeated for 3 times (n=3).

Downregulation of MiR-141 Increased EGFR, MAP2K4 and E2F3 Expression

Our study demonstrated that down-regulation of mir-141 increased expression level of EGFR (5.7fold), MAP2K4 (10.6 fold) and E2F3 (6.7 fold) in cells transfected with mir-141 inhibitory vector; however, MAP2K4 expression fold change is greater. Data was normalized against GAPDH and was statistically significant ($p < 0.05$).

Effect of MiR-141 Ectopic Overexpression on EGFR, MAP2K4 and E2F3 Expression Level

In contrast to the preceding group of cells, expression level of EGFR, MAP2K4 and E2F3 was not statistically significant in miR-141 overexpressed cells or control group. However, E2F3 expression showed a greater fold change.



Research Article

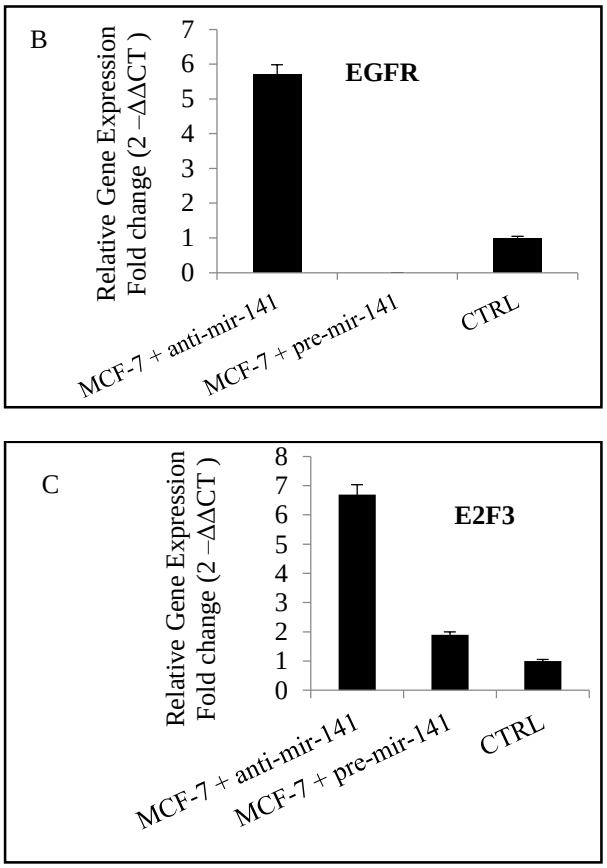


Figure 4. Expression level of predicted target genes in transfected cells by real time-PCR. A) E2F3, B) EGFR, C) MAP2K4

Figure 4 shows the relative expression changes of MAP2K4, EGFR and E2F3 genes in untreated cells as control group as well as transfected cells with pre-mir-141 and anti-mir-141 vector.
Data is normalized with GAPDH as housekeeping gene. For relative quantification of gene expression each test has been repeated for 3 times (n=3).

Network Construction, Module Finding and Enrichment Analysis

PPI Network Construction

PPI Network A encompasses 460 nodes and 464 edges (Figure 5), compared with 803 nodes and 969 edges in PPI network B (seed protein =47) (Figure 7).
A subnetwork containing 10 nodes of degree of 2 or higher and 14 edges is derived from network A (Figure 6). Sub network B contains 152 nodes and 318 edges. Proteins with the highest degree nodes are shown in figure 8.

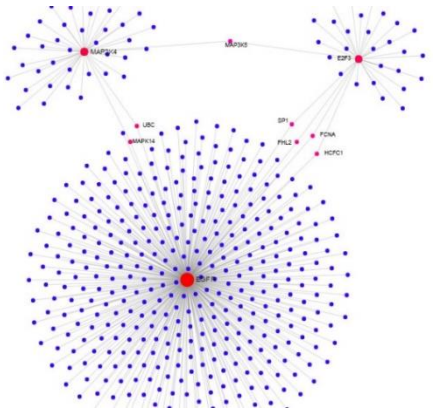


Figure 5. PPI total network A

Figure 5 shows nodes and edges in network A. EGFR is a hub protein with highest degree node.

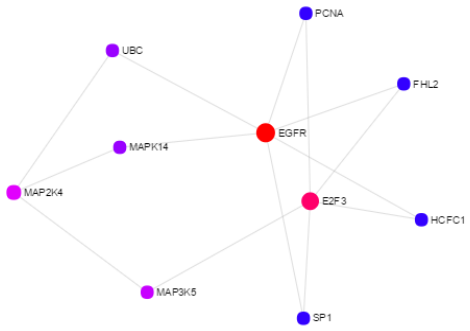


Figure 6. Subnetwork A

From subnetwork A that is retrieved from network Aproteins with the highest betweenness and degree are represented in figure 6. PPI network B contains 803 nodes and 969 edges. Red circles are hub proteins.

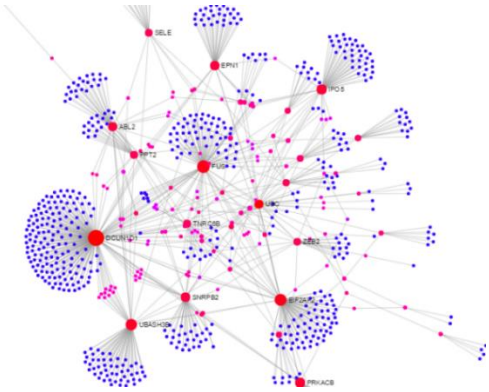


Figure 7. PPI total network B

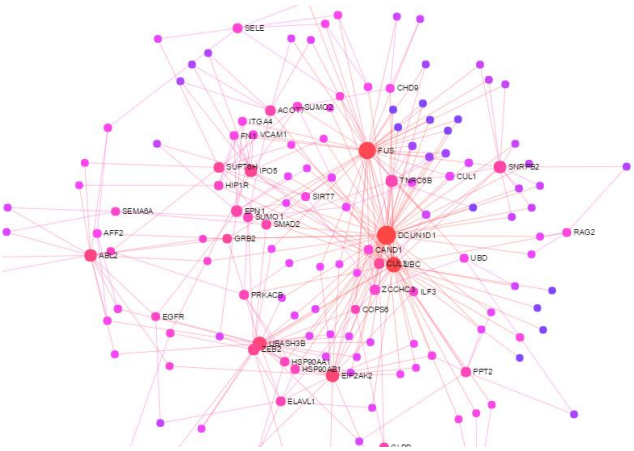


Figure 8. Subnetwork B, derived from network B

Proteins with the highest degree nodes are shown as red color circles in figure 8. Sub network B contains 152 nodes and 318 edges.

Module Analysis in the Network

Three functional modules are explored for each network with connection-first approach algorithm of networkanalyst. When Functional Pathway Enrichment Analysis is sorted by p-value, total network A shows the lowest p-value in immune system (P-value = 0), signaling by interleukins (P=7.19E-28) and innate immune system pathways (P-value = 1.11E-27).

The largest module in Network A was enriched in response to immune system (p-value = 1.19E-28), signaling by EGFR in cancer pathways (P-value = 1.13E-29). Pathways with the highest hits in this module include immune system (P-value = 1.13E-29, 113 hits), signal transduction (P-value =7.35E-10, 101 hits) and adaptive immune system pathways (P-value = 1.59E-17, 68 hits). Interestingly, in both cases when data are sorted by p-value and number of hits, the second detected module in network A shows its most relationship with immune system pathways such as Toll Like Receptor 10 (TLR10) Cascade (P-value =6.06E-17, 12 hits)., Toll Like Receptor 5 (TLR5), Cascade (P-value= 6.06E-17, 12hits), MyD88 cascade initiated on plasma membrane (P-value= 6.06E-17, 12hits). In spite of the two previous modules, the third one was enriched in response to cell cycle pathway (P-value= 6.68E-09, 9hits).

Total network B with function first approach of networkanalyst module explorer enriched to Processing of Capped Intron-Containing Pre-mRNA (P-value= 4.04E-23, 54 hits) and immune system (P-value = 1.94E-20, 155 hits) respectively. In Subnetwork B, proteins with highest degree nodes are enriched in response to Developmental Biology (P-value= 1.01E-07), Immune System (P-value=5.64E-07), Adaptive Immune System (P-value=6.47E-07), Cytokine Signaling in Immune system (P-value= 1.28E-06) respectfully. Moreover, sorted Pathways with the highest hits in this network are similar to pathways sorted by P-value; Immune System (P-value=5.64E-07, 27 hits), Signal Transduction (P-value= 0.00222, 26 hits), Adaptive Immune System (P-value=6.47E-07, 20 hits), Developmental Biology (P-value=1.01E-07, 17 hits).

The largest detected module in Network B with connection-first approach is enriched in response to mRNA Splicing (*P-value*= 7.63E-15), mRNA Splicing - Major Pathway (*P-value*= 7.63E-15), Processing of Capped Intron-Containing Pre-mRNA (*P-value* = 1.42E-14), mRNA Processing (*P-value*= 1.89E-14); however, when enriched pathways are sorted with hits, proteins in Gene Expression (*P-value*=1.01E-07, 17 hits), disease (*P-value*=1.01E-07, 17 hits) and Immune System pathways (*P-value*=1.01E-07, 17 hits) are targeted significantly. Top hits pathways in the second module of the network B are Gene Expression (*P-value*= 7.43E-11, 29 hits), Immune System (*P-value* = 0.00162, 18 hits), Adaptive Immune System (*P-value*= 8.86E-05,15hits), Signaling by NGF (*P-value*= 1.05E-05, 11 hits) and downstream signaling with B cell receptor (*P-value*= 6.49E-07, 11hits). Top hits pathways of the third module are also related to immunity comparatively. These pathways are as follows: Immune System *(P-value* = 0.000133, *17hits)*, Cytokine Signaling in Immune system (*P-value*= 5.60E-07, 11 hits), Disease (*P-value*= 0.0199, 11 hits), Innate Immune System (*P-value* = 0.00306, 9 hits) and Signaling by Interleukins (*P-value*= 4.62E-06, 7 hits).

Discussion

Most of miRNAs in cancer research have been categorized according to the mechanism they are involved, i.e. metastatic, oncogenic, apoptotic. MiR-200 family members are usually categorized as metastamirs. They are responsible for tumor progression, metastasis, invasion and treatment responses (Taylor and Schiemann, 2014). In different types of cancer, intracellular miRNAs are underlying cause of regulation of many caretaker and gatekeeper genes. Adding this to the mechanisms controlled by exosomal miRNA, thousands of cellular and extracellular components is under miRNA's charge.

Among members of miR-200 family, fewer studies have been attributed to miR-141. There have been some well researches documented with regard to the importance of miR-141 in breast cancer. As it has been pointed out in Neves et al, not only MiR200c/141- ZEB1/2 feedback loop, but also methylation of miR-141 promoters is responsible for EMT phenotype of breast cancer (Neves et al., 2010). On the other hand, circulating miR-141 in blood of breast cancer patients has been reported to inform us of many critical features including survival, responses to drugs and metastatic stages (Antolin et al., 2015).

To draw a comparison between our study and other researches that investigate targeting of Map2K4 by miR-141, we found similar evidences in Marasa et al study in ovarian cancer. Since our results is consistent with in this study, it can be concluded that in loss of miR-141, cancer cells are tend to proliferate by a signaling pathway that is triggered by MAPK (Marasa et al., 2009). Moreover, a same mechanism is reported in Wang et all study in pancreatic cells; considering that the mechanism was attributed to another MAPK signaling molecule, MAP4K4, which is functionally the same as MAP2K4 (Wang et al., 2004).

Given that mutation or any gene expression changes in EGFR will lead to resistance to tyrosine kinase inhibitors and undesirable therapeutic consequences (Gazdar, 0000), increased expression of EGFR followed by down-regulation of miR-141 should be taken into account as a notable finding that have to be investigated more in pharmacogenomics studies.

In the current study apart from investigating EGFR, MAP2K4 and E2F3 genes as potential targets for miR-141, which is the first study that suggests targeting of these three genes in MCF-7 cell line, we construct PPI networks to hypothesize other pathways in breast cancer. Accordingly, we find out that in either of networks, although seeded by different targets, enriched pathways of the two PPI networks and detected modules has shown the greatest relationships with innate immunity responses. Experimental studies in different cancer cell lines and tissues also revealed evidence of targeting of immune system by miRNAs; interfering with immunological synopsis in tumor microenvironment, downregulation of MHC1, deregulation of CTL activities as well as differentiation of tumor associated, to name but a few (Rusek et al., 2015).

In glioma cells, miR-29 has proved to downregulate ICAM1.Therefore ICAM can no longer attach to LFA1, hence dysregulation of cytotoxic T cells activities (Ueda et al., 2009). Moreover, in breast cancer PD-L1 is indirectly targeted by miR-200 by TGFβ. PD-L1 by providing a negative feedback causes CTL exhaustion. Chen et.al has shown PD-L1 suppression by miR-200 upregulation. Interestingly, PD-L1 is a mesenchymal marker that is increased in EMT (Chen et al., 2014).

Increased expression of Th17 and decreased expression of regulatory T cells by miR-141 and miR-200 in Multiple- sclerosis patients in relapsing phase has been also reported (Naghavian et al., 2015). Nevertheless, this is the first study that specifically suggests relationships of immune system mechanism with miR-141 in a systematic way. On the other hand, when retrieving high degree nodes proteins in subnetworks retrieved from PPI network A and B, we detect UBC protein from subnetwork A and subnetwork B. These common nodes are related to ubiquitin systems. Interestingly, based on biological process gene ontology term, UBC or Polyubiquitin-C is responsible for common pathways to the pathways associated with target genes we have selected in our study.

These include, MAPK activity, cell surface receptor signaling pathway and antigen presenting antigen processing and presentation of peptide antigen via MHC class I.

Considering the analogy of these pathways, the correlation of breast cancer development by employing components of innate immune system can be hypothesized.

It has to be considered that this in-vitro study has confirmed EGFR, MAP2K4 and E2F3 as the exact target of miR-141 in transcriptome level; however, further research is needed to corroborate the mechanism by proteomics study.

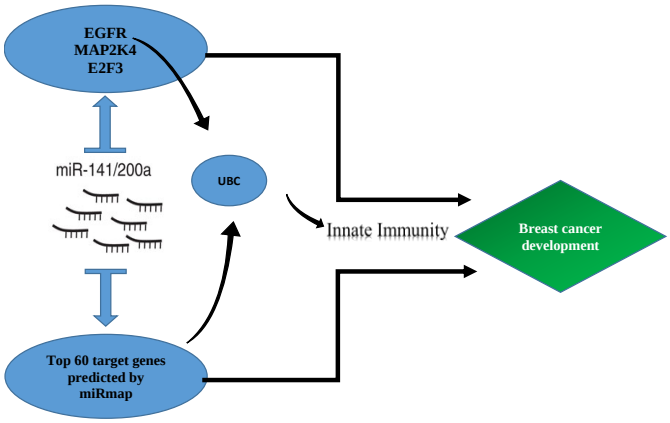


Figure 9. Proposed regulatory mechanisms by miR-141 in breast cancer development through immune system pathway.

Enrichment analysis showed that Both Network A and B take innate immunity signaling molecules as chief molecules by which they control breast cancer development. Note that flashes from UBC to innate immunity do not infer that the only related protein with immune pathway is UBC. UBC is the common protein of the two network, although there are many components that regulate this core pathway. As described previously, UBC protein is one of the highest degree nodes which is retrieved from both of networks. On the other hand, UBC itself have shown to be strongly related to cancer development through immune system pathway and it is structurally interacted with EGFR.

Acknowledgements

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Conflict of Interest

Author Arshad Hosseini has received research grant number 25802 from Iran University of Medical Sciences. Author Soheila Shokrollahzade has no conflict of interest. Author Shamim Sarhadi has no conflict of interest. Author Majid Safa has no conflict of interest.This article does not contain any studies with human participants or animals performed by any of the authors.

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Research Article

RNAseq Reveals Novel and Differentially Expressed Isoforms In Native and Commercial Poultry

Ayeh Sadat Sadr[1], Mohammadreza Nassiri[2*], Seyed Alireza Salami[3], Mohammad Reza Bakhtiarizadeh[4], Mojtaba Tahmoorespur[2], Alireza Shafeinia[5]

[1] *Department of Animal Science, International Campus, Ferdowsi University of Mashhad, Mashhad, Iran*
[2] *Department of Animal Sciences, Faculty of Agriculture, Ferdowsi University of Mashhad, Mashhad, Iran*
[3] *Department of Horticulture, Faculty of Agriculture, University of Tehran, Tehran, Iran*
[4] *Department of Animal and Poultry Science, College of Aburaihan, University of Tehran, Pakdasht, Tehran, Iran*
[5] *Department of Biotechnology, Faculty of Agriculture, Ramin Agriculture and Natural Resources University, Ahwaz, Iran*

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Abstract

The poultry industry occupies an important position in the provision of animal protein. Recently, next generation sequencing technology (RNA-Seq) has become available as a powerful tool to investigate transcriptional profiles for gene expression analysis of many organisms. The main use of RNA-Seq in agriculture species are focusing on finding the immune related genes or pathways by comparison of the whole transcriptome following pathogen challenge. Alternative splicing (AS) is the major fundamental mechanism generating the protein diversity and regulating the gene expression in eukaryotic organism. Identifying genes that are differentially spliced between two groups of RNA-sequencing samples is interesting subject in transcriptome with next-generation sequencing technology in this study used RNA sequencing to comparison isoforms of two breeds. A total of 64,819 transcripts were identified by aligning sequence reads to genome among the evaluated isoforms for expression analysis, 310 were significantly differentially expressed between two breeds, including 251 up-regulated and 59 down-regulated. The KEGG results of up regulated isoforms showed that that no pathway was found significantly different (FDR ≤ 0.05). However, enrichment analysis suggested that seven were over-represented (P-value ≤ 0.05) within the up regulated isoforms. Only one of them functionally related to immune system, natural killer cell mediated cytotoxicity. The results showed genes which are breed-specific expression and the comparative transcriptome analysis help to understand the difference of genetic mechanism.

Keywords: Isoforms, RNASeq, Poultry

Introduction

The poultry industry occupies an important position in the provision of animal protein (meat and egg) to man as well as manure for crops and generally plays a vital role in the national economy as a revenue provider and provides employment (Mohammed and Sunday, 2015). Economic pressure on the modern poultry industry has directed the selection process towards fast-growing broilers that have reduced feed conversion ratio. Selection based on growth characteristics, could adversely affect immune competence leaving chickens more susceptible to disease. Since the innate immune response directs the acquired immune response, efforts to select poultry with an efficient innate immune response would be beneficial (Swaggerty et al., 2009). Indigenous (native) breeds of livestock have higher disease resistance and adaptation to the environment due to high genetic diversity. Therefore, the conservation of diversity in the existing genetic resource is more important for economic and public health than for the development of new breeds with higher productivity (Jeong et al., 2014). Presently, several major issues confront the poultry industry in meeting the growing demands of consumers. Control of infectious disease and food safety is certainly at or near the top of the list (Cheng et al., 2013). The immune system is an adaptive defense system that evolved in phylogenesis to control an organism's integrity (Muir and Aggrey, 2003)

Mammalian pre-mRNA consists of protein coding regions, exons and intervening sequences, introns. The splicing process joins the exon sequences while

Corresponding authors E-mail:
**nassiryr@um.ac.ir*

removing the introns. Recent study revealed that most human genes have alternative splicing and can produce multiple isoforms of transcripts. Differences in the relative abundance of the isoforms of a gene can have significant biological consequences. Identifying genes that are differentially spliced between two groups of RNA-sequencing samples is interesting subject in transcriptome with next-generation sequencing technology (Wang et al., 2013). Alternative splicing (AS) is the major fundamental mechanism generating the protein diversity and regulating the gene expression in eukaryotic organism (Black, 2003; Cáceres and Kornblihtt, 2002). The pre-mRNA undergoes splicing in the nucleus where after removal of intronic sequences, exons are joined in different combinations, leading to generation of isoforms with distinct transcript structure. The proteins thus encoded by transcript isoforms vary in their structures as well as functions. This alteration in the protein structure and function resulting from aberrant or AS is commonly associated with diseases (Tazi et al., 2009). Recently, RNA sequencing (RNA-Seq) has considered a powerful tool to investigate transcriptional profiles of thousands of genes in many organisms (Truong et al., 2015)

Gene expression is a widely studied process and a major area of focus for functional genomics. The main use of RNA-Seq in economical aquaculture species are focusing on finding the immune related genes or pathways by comparison of the whole transcriptome following pathogen challenge (Li and Li, 2014).

In this study, we performed comparative gene expression analysis of native and commercial breed poultry to identify differentially expressed isoforms by RNA-Seq technology. Foundings revealed significant expression differences in isoform expression levels. In addition, we detected novel splicing events and novel transcript structures that are not described previously.

Materials and Methods

RNA Extraction and Sequencing

In total, six chicken from Esfahani and six chicken from Ross breeds (47 days of age) were chosen for RNA-seq. The chicken raised on the farm of Safi Abad Agriculture and education Center Dezful Iran. These birds were kept under the same environmental and nutritional conditions. Five ml blood samples were collected from Brachial/ulnar wing vein. The total RNA was extracted using Trizol (Invitrogen, USA) following the manufacturer's instructions. The RNA pool was prepared by mixing together equal quantities of three RNA samples per group to generate a total of 4 pooled RNA samples (two samples in each breed). The four RNA-seq libraries were sequenced based on protocols of Illumina HiSeq 2000 to generate 150 pair-end reads.

Differentially Expressed Isoforms

The quality of the row data was checked with FastQC vol 0.11.2. Beside on these results, the Trimmomatic (v 0.35) were used to remove Illumina adaptors, trimming of reads as well as quality or filtering reads by removing low-quality reads (Bolger et al., 2014).

The reference genome for chicken (Galgal4) its corresponding annotation GTF file were downloaded from the Ensemble (http://asia.ensembl.org/info/data/ftp/index.html).

Clean data of pair-end reads from each sample were mapped to the reference genome using HISAT2 (v2-2.0.3) (Kim et al., 2015). Cufflinks (v2.2.1) were used to process the alignment files and estimate the abundance of the assembled transcripts as FPKM, (fragments per kilobase of exon per million fragments mapped). Cufflinks were used to normalize the number of fragments mapping to individual loci (Trapnell et al., 2010). Cufflinks includes a script called cuffmerge that can use to merge together several Cufflinks assemblies. Merged.gtf file produced by cuffmerge was provided as an input to cuffdiff along with alignment files produced by Hisat2 for differential analysis between two samples. Also cuffdiff labeled genes as significant or not significant based on the *p-value*. Visualization of expression and differential expression results were performed by CummeRbund package which accepted cuffdiff output (Trapnell et al., 2012).

Functional Annotation

To test for enrichment of GO terms of differentially expressed isoforms, David functional annotation tools (https://david.ncifcrf.gov/) was used. *P-value* reported by David were corrected to obtain FDR (Dennis et al., 2003).

Novel Isoform Detection

Assembled transcripts were annotated using Cuffcompare from Cufflinks. Cuffcompare simply compares the transcripts that have been assembled through Cufflinks to a reference annotation file. To minimize annotation artifacts, all single exon transcripts were excluded for further analysis. Also, all the transcripts smaller than 200 bp were removed.

Cuffcompare classified each transcript as known or novel and identified transcripts that are potential novel isoforms. The class codes in the Cuffcompare output were used to identify novel isoforms. The transcripts with class code "j" (locus is potentially a novel isoform) were considered as novel isoforms of known genes.

According to investigation of the poultry immune system, the tracking file provided by Cuffcompare was used to infer isoforms and splice variants unique to natural killer cell mediated cytotoxicity isoforms by examining manually which related to immune system. Novel splice variants detected by Cuffcompare were annotated manually by UCSC genome browser.

Results

Quality Analysis and RNA-Seq Data

To identify and compare the transcriptome of different chicken breeds, the blood samples were collected from Esfahani as native and Ross as commercial breed. Total RNA was extracted and the RNA pool was obtained. After sequencing, the average number of reads across all 4 samples (2 samples per breed) was approximately 17 million. After removal of low- quality reads, few of the sequence reads did not pass the quality filtering (Table1). Sequence reads were aligned to the chicken reference genome (Galgal4) using HISAT2, approximately 85% of sequenced fragments were aligned successfully (Table1).

Table 1. Summary of sequencing read alignments

	Native		Commercial	
	Sample1	Sample2	Sample1	Sample2
Total read	18768307	17995632	15923838	15621164
Reads after trimming	18509420	17650558	15677357	15313649
Read aligned to reference genome	86.68	84.89	84.36	84.17

Isoform Expression Analysis

After mapping the reads to the reference genome with HISAT2, transcripts were assembled and the relative expression level calculated by cufflinks. Gene expression intensity was estimated using FPKM method. Then the Cuffdiff was used to calculate the differentially expressed isoforms.

A total of 64,819 transcripts were identified by aligning sequence reads to genome among the evaluated isoforms for expression analysis, 310 were significantly differentially expressed between two breeds, including 251 up-regulated and 59 down-regulated (figure1). The differentially expressed isoforms in commercial versus native breed with statistically significant fold changes ranged from -3.50894 to 3.75559. Top ten up and down regulated isoforms were presented in figure 2.

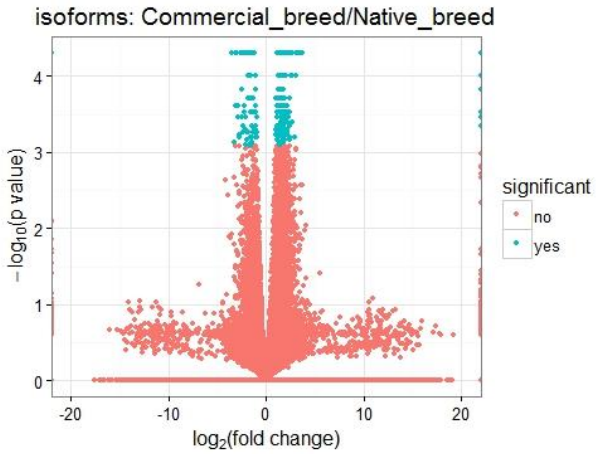

Figure1. Log_2-fold change between the commercial versus native breed**.** Light blue dots represent significantly differentially expressed isoforms between breeds (*P-value* < 0.05).

Pathway Analysis of Differentially Expression Isoforms

To gain insights into the biological processes and pathway that are enriched in differentially expressed isoforms, DAVID was used. Results were grouped in cellular component molecular function and biological process. Top five GO terms identified in up and down regulated isoforms are presented in table2. The KEGG results of up regulated isoforms showed that that no pathway was found significantly different (FDR ≤ 0.05). However, enrichment analysis suggested that seven pathways including focal adhesion, regulation of actin cytoskeleton, lysosome, natural killer cell mediated cytotoxicity, VEGF signaling pathway, glycolysis/gluconeogenesis and ECM-receptor interaction were over-represented (*P-value* ≤ 0.05) within the up regulated isoforms. Only one of them functionally related to immune system, natural killer cell mediated cytotoxicity. Also, the pathway analysis results showed that no pathway were found significantly enriched for down regulated isoforms (corrected *P-value* ≤ 0.05).

Figure2. The most up regulated (n=10) and down regulated (n=10) differentially expressed isoforms between native vs commercial breeds. The differentially expressed isoforms were ranked based on their fold change.

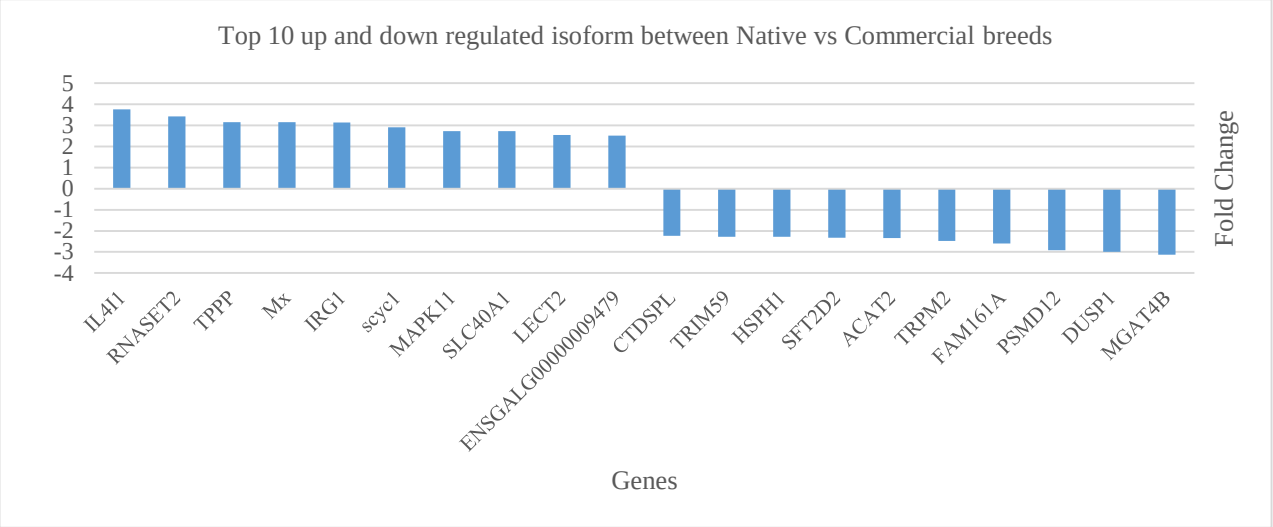

Table2. Top five GO terms significantly enriched in up and down regulated differentially expressed isoforms.

Term	Count	%	P value
Up regulated			
actin cytoskeleton organization	9	4.347826	1.51E-05
biological regulation	63	30.43478	2.48E-05
actin filament-based process	9	4.347826	2.61E-05
signal transduction	30	14.49275	3.54E-05
cytoskeleton organization	11	5.31401	3.65E-05
Down regulated			
histone modification	3	6.382979	0.005163
covalent chromatin modification	3	6.382979	0.005725
chromatin modification	3	6.382979	0.019129
post-translational protein modification	6	12.76596	0.02271
regulation of transcription	7	14.89362	0.031248

Novel Splice Variants

We obtained 64,819 unique alignments that were screened for novel isoforms using Cuffcompare. We found 37,057 isoforms already present in the annotation (corresponding to 10530 distinct genes). Moreover 27,762 novel isoforms were identified (corresponding to 7310 distinct genes), which have not been included in the gene annotation so far. Of these novel splice variants 11,734 were present in native breed, while 13,234 were present in the commercial breed. To gain a better understanding of the genes in immune system the genes in the natural killer cell mediated cytotoxicity pathway were analyzed and four genes with novel isoform were detected (PIK3CG, PLCG2, PTPN6 and PPP3CA) and showed alternative splicing (figure3).

Discussion

In present study, high throughput sequencing was performed to evaluate the transcription of two chicken breeds with emphasis on immune system. The immune system plays a key role in health maintenance and pathogenesis of a wide range of disease.

In this study the Ross as commercial and Esfahani as a native breed were used with difference in same traits. To achieve accurate detection of splicing variants and to optimally decode mechanisms underlying alternative splicing, it is invariably important to accurately map cDNA reads to their genomic counterparts.

Figure3. Blast analysis showing alternative splicing. Splice variants identified in different expressed genes with novel isoform in Natural killer cell mediated cytotoxicity pathway after submission of transcripts assembled by Cuffcompare as custom tracks to UCSC genome browser.

It is also important to note that an aligner for RNA-Seq data should efficiently align reads matching to splice junctions (Li and Homer, 2010). Here, we used an efficient aligner, HISAT2, to identify differentially expressed isoforms as well as novel isoforms. Until now, identification and differential expression analysis of genes involved in immune system has not been reported by comparison between two different breeds in poultry. In our best knowledge, this is the first study to identify differentially expressed isoforms between two chicken breeds. However, the different studies were carried out on poultry which most of them emphasis on disease (Perumbakkam et al., 2013; Sandford, 2011; Wang et al., 2014).

As a results of mapping with HISAT2, 85.8% and 84.3% of reads were mapped in native and commercial breed respectively. Through comparison of the transcriptome data of the two breeds, 310 isoforms were significantly differentially expressed, which 251 isoforms were up-regulated in the commercial vs native breed and 59 genes were down-regulated in the native vs commercial breed. KEGG pathway analysis results showed that one immune related pathways was enriched among up-regulated isoforms (*P-value* $\leq$ 0.05), Natural killer cell mediated cytotoxicity with seven genes including PIK3CG, PTPN6, VAV3, RAC2, PLCG2, PIK3CD and PPP3CA. Natural killer (NK) cells are lymphocytes of the innate immune system that are involved in early defenses against both allogeneic (no self) cells and autologous

cells undergoing various forms of stress, such as infection with viruses, bacteria, or parasites or malignant transformation. One of the important aspect of RNA-Seq is discovered novel exon and novel exon boundaries. So that for this analysis all genes related to natural killer cell mediated cytotoxicity were analyzed. PIK3CG, PTPN6, PIK3CD and PPP3CA were detected as novel isoform. For splicing variants in these genes, the Cuffcompare as custom tracks to the UCSC genome browser was submitted and the blast was carried out. The blast results showed the different types of alternative splicing like exon skipping, exon insertion, 5 alternative splicing and intron retention which may change the protein sequence. For example the intron retention was observed in PTPN6 gene (figure3). The alternation in the protein sequence may thus lead to either gain of function, loss of function or change in the specify of the protein and its functional diversity. The results demonstrate the ability of RNA-Seq to detection of novel isoform.

Our study is the first investigation of poultry transcriptome to compare two different breeds in normal situation by using high throughput sequencing. The results showed genes which are breed-specific expression and the comparative transcriptome analysis help to understand the difference of genetic mechanism. In conclusion, the results of this study would be used to recognize gene candidates for further breed improvement.

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Research Article

Investigation of Genetic Variation In *Berberis Vulgaris* Using ISSR and SSR Molecular Markers

Behnaz Safamanesh[1], Sedigheh Esmaeilzadeh Bahabadi[1*], Ali Izanloo[2]

[1] *Department of Biology, Faculty of Basic Sciences, University of Zabol, Zabol, Iran*
[2] *Department of Agronomy, Faculty of Agriculture, University of Birjand, Birjand, Iran*

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Abstract

Barberry fruit is a medicinal plant, and it is one of the most important horticultural crops in South Khorasan province, Iran. Genetic diversity has a basic role in the successful breeding of crop varieties with durable resistance to biotic and abiotic stresses. The main objective of this study was to assess the genetic diversity of 20 ecotypes of *Berberis*, which collected from different regions of South Khorasan province, Iran, using ISSR and SSR markers. In this study, 10 ISSR primers and 5 SSR primer pairs, with the amplification of suitable polymorphic alleles were used. A total of 98 bands for ISSR markers and 43 bands for SSR markers were detected between 300 to 1300 bp and 100 to 1100 bp in size, respectively. Polymorphic ISSR-7 and CA03 primers amplified the highest number of alleles with 17 and 24 bands, respectively, while ISCS50 and CA30 primers amplified only two polymorphic alleles. The ISCS57 and GA31 primers had the highest polymorphic information content (PIC) and ISCS50 and GA04 primers had the lowest PIC. The estimated Nei's and Shannon indices for genetic diversity in ISSR markers were 0.24 and 0.35, while for SSR markers these were 0.23 and 0.34, respectively. Based on cluster analysis, five and six main groups were identified for ISSR and SSR markers, showing high genetic variations among a set of collected barberry ecotypes. Analysis of molecular variances in both ISSR and SSR markers showed that high level of total variation was due to within populations, rather. Therefore, it will be better to select within populations in breeding programs.

Keywords: *Berberis* spp, Genetic diversity, ISSR, SSR

Introduction

Barberry (*Berberis* L.) is a well-known medicinal plant, which has been used for a long time in Iran and many other ancient civilizations around the world (Heidary et al., 2009). This plant is a deciduous, evergreen and semi-evergreen shrub which grows up to 4 m high and under a wide range of ecological conditions (Bottini et al., 2000; Rezvani Moghaddam and Koocheki, 2007). Barberries are mostly diploid (2n = 2x = 28) and few are tetraploid (Cadic, 1992).

They are self-fertile and mainly autogenous (Rezaei et al., 2011). They are vegetatively and sexually propagated plants (Bottini et al., 2002). *Berberis* is the largest genus in the Berberidaceae family (Kim et al., 2004).

The genus includes about 450-500 species that grows in Asia, Europe and America. *Berberis* is a well-known medicinal plant in Iran (Sodagar et al., 2012; Shamsa et al., 1999) and five species have been reported so far, which include *B. vulgaris* L., *B. orthobotrys* Bien. ex Aitch., *B. crataegina* DC., B. *integerrima* Bunge and *B. khorasanica* Browicz and Ziel. (Alemardan et al., 2013; Rezaei and Balandary, 2015; Tavakoli et al., 2016).

Seedless barberry is one of the few unique crops cultivated only in Iran, especially in South Khorasan province (Heidary et al., 2009). In Iran, this plant occupies approximately 95% of the total cultivated area and production of this plant is located in these regions (Heidary et al., 2009).

Berberis has been reported as a tolerant plant to low temperature, drought, and wind (Varas et al., 2013). Due to salinity stress and water scarcity, most of the agricultural land in South Khorasan are not suitable for the growth of most of the other crops, hence, the seedless barberry has been introduced as a major crop, during the last 20 years. Cultivation and production of seedless barberry took place in Afin village in Ghayenat, Zirkooh district for the first time. Until 50 years ago, seedless barberry was

Corresponding authors E-mail:
** esmaeilzadeh@uoz.ac.ir*

mainly cultivated in Afin and Darmian villages, however, ever since seedless barberry has been cultivated in most of the villages and regions (Javadzadeh and Fallah, 2012). Nowadays, there are over 11,000 ha of cultivated areas under seedless barberry, with a production of more than 9200 tons of dried fruit per year (Alemardan et al., 2013).

The knowledge of genetic diversity is an essential tool in gene-bank management and breeding experiments. The assessment of genetic diversity and the characterization of germplasm are prerequisite to improve the chances of selecting better segregants for various characters (Dwevedi and Gaibriyal, 2009).

Determination of genetic diversity can be based on morphological, biochemical and molecular markers (Mohammadi and Prasanna, 2003; Sudre et al., 2007; Goncalves et al., 2009). However, the huge diversity, polyploidy levels, spontaneous mutations and subsequent recombinations make the identification of the species rather difficult and controversial.

The origins and relationship between seedless types have remained unclear so far. Seedless barberry has often been considered as *B. vulgaris* var. asperma in Iran as well as in Europe, but some authors described it as "*B. vulgaris* Asperma" (Hatch, 2007; Azadi, 2009).

However, recent studies indicated that Iranian seedless cultivar belongs to B. integerrima (Rezaei et al., 2011; Alemardan et al., 2013). Assessment of the relationships between seeded and seedless barberry and evaluation of genetic diversity will provide useful information to breeders.

So far, all studies have been focused on medicinal properties of barberry and little is known about the genetic diversity of seedless barberry cultivars and its wild-type relatives.

Therefore, the main objective of this research was to study the genetic diversity of barberry ecotypes (seeded and seedless) collected from South Khorasan province using ISSR and SSR markers.

Materials and Methods

Plant Materials

In this study, 20 barberry ecotypes selected from different regions of South Khorasan province, were evaluated genetically, using ISSR and SSR molecular markers.

Among these ecotypes, 11 samples were seeded-barberry with red color berries, three samples were seeded-barberry with black color berries and six were seedless cultivated barberry (Table 1).

Young leaves of selected ecotypes were harvested in pril 2015. The collected samples were kept in ice and transferred to the laboratory snap frozen in liquid nitrogen, and then stored at -20°C until the DNA extraction.

Genomic DNA Extraction

DNA was extracted according to CTAB method (Doyle and Doyle, 1990), with minor modifications. The quantity and quality of the extracted DNA were assessed using Nano Drop 2000 spectrophotometer and agarose gel electrophoresis 0.8%. The extracted DNA was then diluted to 15 ng/µl as working concentration for PCR.

ISSR and SSR Analysis

A total of 10 ISSR primers and 5 primer pairs of SSRs developed by Roß and Durka (2006) were selected and used for PCR amplification of the DNA templates (Table 2).

PCR reactions were performed in a 20 µL volume mixture in a gradient thermal cycler (Master cycler® gradient, Eppendorf, Hamburg, Germany).

The PCR reaction mixture for ISSRs contained 1.5 µL of the 10 µ mol/l primers, 10 µL of PCR ready Master Mix (CinnaGen Co., Iran), 2 µL of template DNA (30 ng), and 6 µL of sterile water.

For SSRs, however, the reaction mixtures included 1 µL of the 10 µmol/l of each forward and reverse primers, 10 µL of PCR ready Master Mix (CinnaGen Co.), 3 µL of template DNA (45 ng), and 5 µL of sterile water.

The following PCR program was used for ISSR marker: 3 min at 94°C; followed by 35 cycles at 94°C for 30s, 43- 61°C (depending on the Tm of the primers) for 45s and 72°C for 1 min; then left at 72°C for 10 min.

For SSR marker, however, the following PCR program was set up; initial denaturation step at 94°C for 3 min, followed by 35 cycles of 94°C for 35s, annealing for 60s (optimized temperature between 57°C and 62°C, depending on the primer pairs) and extension at 72°C for 90s with a final extension at 72°C for 10 min.

The PCR products were separated by electrophoresis on 2% agarose gel in 0.5x TBE buffer, stained with ethidium bromide, and subsequently visualized using UV lights. A DNA size marker of 100 bp (DENAzist Asia Co., Iran) was used to estimate the size of different bands amplified for each primer or primer pairs. Amplification experiments repeated twice to confirm the band amplification results.

Table 1. The list of 20 barberry ecotypes evaluated in this study with their location names and fruit and leaf characteristics

Number ecotype	Name ecotype	Location	Color and shape of fruit	Shape and Color of leaf	Fruit image	Leaf image
1	Seeded	Birjand	Round and stretched dark red	Rhombus stretched with short prickles around leaf dark green		
2	Seeded	Ghehardeh	Stretched Dark red	Elliptic without prickles around leaves Light green		
3	Seeded	Razg	Round and stretched light red	Elliptic and flattened with very small prickles around leaves Light green		
4	seedless	Birjand	Round and stretched light red	Rounded and stretched with very small prickles around leaves Light green		
5	black seeded	Behdan	Ovate Black	Elliptic stretched with prickly edge dark green		
6	Seeded	Behdan	Round and large Red	Elliptic and flattened with prickly edge dark green		
7	black seeded	Behdan	Round and stretched Black	Elliptic and flattened, Prickles around leaf to each other over long distances dark green		
8	black seeded	Behdan	Round Black	Rounded and flattened with very small prickles around leaves dark green		
9	Seeded	Behdan	Round Red	Elliptic and flattened with prickly edge Light green		
10	Seeded	Behdan	Round Pink	Elliptic and flattened with prickly edge dark green		
11	Seeded	Behdan	Round and large Dark red	Rounded, Prickles around leaf to each other over long distances dark green		
12	Seeded	Behdan	-	Rounded and flattened with Long prickles around leaves dark green, Around the red leaves	-	
13	Seeded	Behdan	Stretched light red	Elliptic stretched with edge without prickles dark green		
14	seedless	Behdan	Round and stretched light red	Elliptic and flattened with very small prickles around leaves dark green		
15	Seeded	Behdan	Round and Ovate Dark red	Rhombus and flattened with Long prickles around leaves Light green		
16	Seeded	Behdan	-	Rounded and stretched with Long prickles around leaves dark green	-	
17	seedless	Noghab	Round light red	Elliptic and stretched with edge without prickles dark green		
18	seedless	Nozad	Round and large Red	Elliptic and flattened with very small prickles around leaves Light green		
19	seedless	Dorokhsh	Round and large Red	Rhombus and flattened with prickly edge dark green		
20	seedless	Afin	Stretched light red	Elliptic and stretched flattened with prickly edge dark green		

Data Analysis

Each of the DNA fragments amplified by ISSR and SSR primers were scored 1 for presence and 0 for absence of the specific bands. The polymorphic information was calculated for all the primers (Equation 1).

$$PIC = 1 - \sum_{i=1}^{n} p_i^2 - \sum_{i=1}^{n-1} \sum_{j=i+1}^{n} 2 p_i^2 p_j^2 \quad (1)$$

In which, *pi* is the *i*th allele frequency for co-dominant markers and *i*th band frequency for dominant markers (Anderson et al., 1993; Powel et al., 1996).

These similarity coefficients were estimated according to Jacard (J) index (Nei and Li, 1979). Cluster analysis was performed based on the unweighted pair group method with arithmetic averages (UPGMA) algorithm and employing the sequential, agglomerative, hierarchical, and nested clustering (SAHN) using NTSYS PC2.02 program (Rohlf, 1998).

To determine the genetic relationships between the studied ecotypes and to categorize them, a principal coordinate analysis (PCoA) as a complementary method for cluster analysis (Huff *et al*., 1993) was also performed using the same program.

Barberry ecotypes collected were grouped According to the geographical collection site in three populations named as Birjand (including seedless, seeded and black seeded barberry Birjand, Ghehardeh, Razg and Behdan), Ghayenat (including seedless barberry Dorokhsh and Afin) and Darmian (seedless barberry Noghab and Nozad).

Analysis of molecular variance (AMOVA) to determine the amount of genetic variation within and between populations was performed using GenAlEx version 6.1 (Huff et al., 1993; Excoffier et al., 1992). Genetic coefficients and indices such as number of polymorphic loci, percentage of polymorphic loci, observed number of alleles (Na), effective number of alleles (Ne), Nei's gene diversity (*h*), Shannon's Information index (I), heterozygosity within populations (Hs), total heterozygosity between populations (Ht), Gst Factor and Nm of gene flow were analyzed using the POPGENE program version 1.32 (Yeh *et al*., 1997). Differences among populations were quantified using Wright's inbreeding coefficient (Fst).

Fst = 1-HS / HT

Table 2. ISSR and SSR primers used in the present study

Name marker	Primer seq. (5'- 3')	Annealing temperature (°C)
ISCS1	5' - (TCC) $_6$ C - 3'	61
ISCS7	5' - (TC) $_9$C - 3'	48
ISCS50	5' -(CA) $_8$ RC - 3'	47
ISCS57	5' -(GA) $_8$ YG - 3'	39
ISCS80	5' -(TG) $_8$ C - 3'	51
ISSR2	5' -(AG) $_8$ YT -3'	46
ISSR3	5' -(AG) $_8$ T - 3'	45
ISSR4	5' -(GA) $_8$ T - 3'	45
ISSR6	5' -DBD (AC) $_7$ - 3'	43
ISSR7	5' -(AG) $_8$ RC - 3'	49
GA33	F: GAT CAG GTC CAT AAT ATC AAA GTT C (25mer) R: CAG ACA AGG AGA GTG CTT GTA CC (23 mer)	62
CA03	F: GGG GTG TGA CCG TTT TTA TG (20 mer) R: CAA TGC CCG AAA GTT ACG TC (20 mer)	58
CA30	F: TGC ATT TTC GAC CCA TCT AC (20 mer) R: TCT CCT CAC ATG CAA CAA AAG (21 mer)	57
GA04	F: ACC CAT TGG AGC TCT CTC AG (20 mer) R: TTG ATT TTG AAG CCG AGA TG (20 mer)	57
GA31	F: TCA CAA TAG TTT ATT TGA GTT TAT TTG (27 mer) R: CAC TGT CTG GCT CAA TTT TGT C (22 mer)	57

Results

Genetic Parameters Based on ISSR Markers

In this study, 55 ISSR primers were initially screened for DNA amplification of tested barberry ecotypes using a gradient PCR. Among the tested ISSR primers, only 10 primers produced reproducible bands across 20 barberry ecotypes (Figure 1).

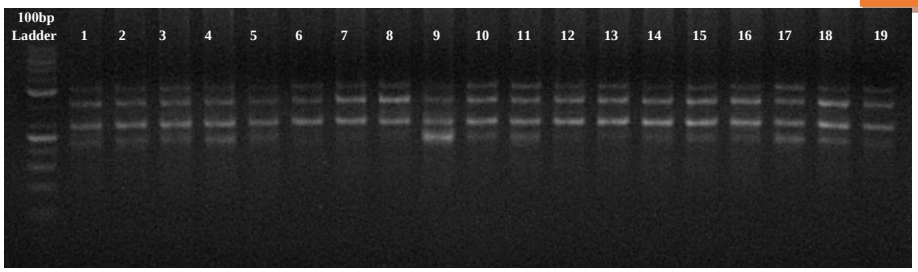

Figure 1. Amplified fragments of ISCS80 for 19 ecotypes

A total of 98 bands were detected using these 10 ISSR markers, out of which 12 were monomorphic and 86 were scorable polymorphic bands (Table 3). The number of amplified fragments varied from 3 (ISCS 50) to 18 (ISSR 4), with an average of 9.8 bands per primer. The maximum number of polymorphic bands (18 bands) were observed for ISSR 4; the average number of polymorphic bands was 8.6 per primer. The size of ISSR bands obtained varied from 30 to 1500 bps (Table 3).

Table 3. ISSR and SSR primers used in the study including number of total bands (NT), size range of amplified fragments (SR), number of polymorphic bands (NP), percentage of polymorphic fragment (PP) and polymorphic information content (PICavg) of 10 ISSR primers and 5 SSR primer pairs.

Name Primer	NT	SR	NP	PP (%)	PIC_{avg}
ISCS50	3	500 - 800	2	66.66	0.56
ISCS57	5	500 - 750	4	80	0.72
ISCS7	11	400 - 900	11	100	0.68
ISCS80	4	500- 800	1	25	0.68
ISCS1	8	400 - 700	8	100	0.71
ISSR2	6	400 - 1000	4	66/66	0.61
ISSR3	13	300 - 1200	10	76.92	0.65
ISSR4	18	350 - 1000	18	100	0.61
ISSR6	13	350 - 1150	11	84.61	0.69
ISSR7	17	400 - 1300	17	100	0.68
Mean	9.8	-	8.6	79.98	0.65
GA33	12	500 – 900	12	100	0.35
CA03	24	300 - 1100	24	100	0.38
CA30	2	100 - 220	2	100	0.4
GA04	3	100 - 300	3	100	0.33
GA31	2	100 - 300	2	100	0.46
Mean	8.6	-	8.6	100	0.38

The average of total heterozygosity between populations, heterozygosity within populations, diversity among populations (Gst), the fixation Index (Fst) and gene flow (Nm) were 0.30, 0.15, 0.96, 0.47 and 5.65, respectively (Table 4). The highest (1.22) and lowest (0.23) total of heterozygosity were observed for ISCS1 and ISCS7 primers, respectively. ISCS50 and ISSR6 primers had the highest (0.21) and lowest (0.07) heterozygosity within populations.

The primer ISCS50 also showed the highest coefficient of variation between populations with 6.34, while the lowest value for coefficient of variation between populations was observed for ISCS1 with 0.1. Gene flow and Fst ranging from 37.69 (ISCS1) to 0.98 (ISCS80) and 0.62 (ISSR6) to 0.13 (ISCS1), respectively.

When individuals in a population are quite similar in terms of allelic frequency, Fst values is equal to 0, when they possess different alleles, this value would be equal to one (Holsinger & Weir, 2009).

One of the important indicators for genetic diversity is the number of alleles detected at each position for the studied individuals (Nevo, 1978). The average number of alleles (Na) and the number of effective alleles (Ne) were 1.37 and 1.19 respectively (Table 4).

The Na value ranged from 1.08 (ISCS80) to 1.62 (ISCS1) and the value for Ne ranged from 1.08 (ISCS80) to 1.32 (ISCS1). The mean Nei's genetic diversity index (H) and Shannon index (I) were 0.24 and 0.35, respectively. The highest Nei's genetic diversity index and Shannon index were observed for ISCS1 primer with the value of 0.19 and 0.30, respectively whereas the lowest Nei's genetic diversity index and Shannon index were observed for ISCS80 primer, with 0.04 and 0.05, respectively.

Genetic parameters based on SSR markers

All five SSR markers showed reproducible bands with three to five amplified fragments in all 20 barberry ecotypes. In total, 43 alleles were identified by five primer pairs (Table 3).

Table 4. Genetic diversity data and differentiation parameters from ISSR and SSR molecular markers for three natural populations of barberry in South Khorasan province. Number of total heterozygosity between populations (Ht), heterozygosity within populations (Hs), Diversity among populations (Gst), Fixation Index (Fst), Gene flow (Nm), observed alleles (Na), Number of effective alleles (Ne), Nei's gene diversity (H), Shannon's information index (I).

Primer	Ht	Hs	Gst	Fst	Nm	Na	Ne	H	I
Mean (ISSR)	0.30	0.15	0.96	0.47	5.65	1.37	1.19	0.24	0.35
Mean (SSR)	0.29	0.23	0.18	0.22	25.15	1.61	1.4	0.23	0.34

Number of alleles ranged from two (CA30 and GA31) to 24 (CA03), with an average of 8.6 alleles per locus. The overall size of amplified products ranged from 100 bp (CA30, GA04 and GA31) to 1100 bp (CA03). The size difference between the smallest and largest allele at a given SSR locus varied from 120 (CA30) to 800 (CA03). Multiple alleles were observed at a rate of 100% in all ecotypes for the SSR markers. PIC values ranged from 0.33 to 0.46, with an average value of 0.38 per locus. The most informative markers were ISCS57 and ISCS1 with PIC values of 0.72 and 0.71, respectively (Table 3).

For SSR primers, the mean of heterozygosity, heterozygosity within populations, coefficient of variation between populations, the Fst and gene flow were 0.29, 0.23, 0.18, 0.22 and 25.15, respectively (Table 4). The highest and lowest total of heterozygosity were observed for GA31 primer with 0.46 and CA03 with 0.2, respectively. GA31 and GA33 primers showed the highest (0.36) and lowest (0.13) heterozygosity within populations, respectively.

The highest coefficient of variation between populations and Fst were observed for GA33 primer with 0.19 and 0.30, respectively and the lowest coefficient of variation between populations and Fst were observed for GA04 primer with 0.02 and 0.03, respectively. The highest gene flow was recorded for GA04 primer with a value 87.98, while the lowest value for this parameter was observed for CA30 primer with a value of 2.34.

The average number of alleles (Na), number of effective alleles (Ne), Nei's genetic diversity index (H) and Shannon index (I) were 1.61, 1.4, 0.23 and 0.34, respectively (Table 4). The highest number of alleles, the number of effective alleles, Nei's genetic diversity index and Shannon index were observed for GA31 primer with 1.83, 1.65, 0.36 and 0.52, respectively, while the lowest number of alleles, the number of effective alleles, Nei's genetic diversity index and Shannon index were detected for GA33 primer with 1.47, 1.22, 0.13 and 0.21, respectively.

Analysis of molecular variance and cluster analysis based on ISSR and SSR markers

Analysis of molecular variance using ISSR and SSR markers showed that, the greatest variation was related to within populations. Variations within populations were 20 and 11 for ISSRs and SSR markers, while the variation between populations were 80 and 89, respectively (Figure 2).

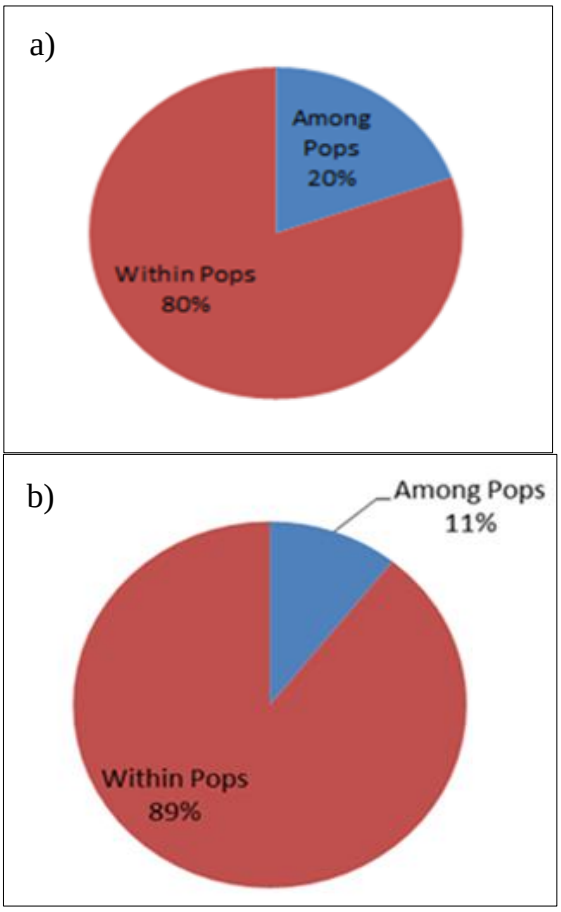

Figure 2. The percentage of molecular variance between and within populations of 20 barberry ecotypes investigated using a) ISSR primers and b) SSR markers

The similarity matrix for ISSR and SSR primers based on the Jacard coefficient for 20 barberry ecotypes were shown in Tables 5 and 6.

Research Article

Table 5. Similarity matrix for the 20 barberry ecotypes investigated using ISSR markers

Name genotype	w	W	w	c	wb1	w1	wb2	wb3	w2	w3	w4	w5	w6	C	w7	w8	c	c	c	c
1- w	1																			
2- w	0.56	1																		
3- w	0.62	0.61	1																	
4- c	0.38	0.31	0.46	1																
5- wb1	0.36	0.32	0.39	0.36	1															
6- w1	0.54	0.42	0.41	0.41	0.46	1														
7- wb2	0.60	0.53	0.58	0.40	0.44	0.53	1													
8- wb3	0.45	0.43	0.37	0.29	0.38	0.43	0.56	1												
9- w2	0.62	0.49	0.54	0.43	0.44	0.53	0.69	0.51	1											
10- w3	0.44	0.45	0.56	0.54	0.38	0.37	0.49	0.53	0.56	1										
11- w4	0.40	0.41	0.46	0.44	0.28	0.38	0.40	0.40	0.35	0.51	1									
12- w5	0.52	0.52	0.48	0.35	0.30	0.40	0.50	0.44	0.42	0.47	0.46	1								
13- w6	0.51	0.51	0.56	0.36	0.36	0.39	0.35	0.43	0.35	0.55	0.54	0.43	1							
14- c	0.44	0.42	0.47	0.51	0.37	0.48	0.38	0.40	0.38	0.43	0.51	0.38	0.55	1						
15- w7	0.36	0.35	0.47	0.32	0.43	0.32	0.33	0.33	0.38	0.35	0.35	0.38	0.40	0.28	1					
16- w8	0.37	0.33	0.34	0.24	0.31	0.33	0.42	0.39	0.36	0.33	0.40	0.36	0.31	0.36	0.32	1				
17- c	0.56	0.50	0.64	0.47	0.37	0.39	0.48	0.48	0.49	0.63	0.53	0.52	0.61	0.48	0.42	0.30	1			
18- c	0.40	0.41	0.53	0.46	0.36	0.35	0.41	0.41	0.42	0.44	0.33	0.32	0.41	0.38	0.47	0.20	0.61	1		
19- c	0.28	0.20	0.31	0.19	0.24	0.23	0.30	0.27	0.28	0.32	0.28	0.19	0.26	0.32	0.18	0.31	0.32	0.24	1	
20- c	0.37	0.29	0.33	0.15	0.19	0.21	0.35	0.25	0.25	0.20	0.25	0.34	0.19	0.24	0.25	0.34	0.30	0.17	0.37	1

According to the results of similarity matrices based on ISSR primer, seeded ecotypes of Behdan (w2) and black of Behdan (wb3) had the highest similarity (0.69), but seedless ecotypes of Birjand and Afin showed the lowest (0.15).

The similarity matrices based on SSR primers have also shown that seeded ecotypes of Behdan (w2) and black of Behdan (wb3) had the highest similarity (0.56), while seeded ecotypes of Behdan (w4) and seedless of Afin represented the lowest similarity (0.04) (Tables 5, 6).

Cluster analysis of the studied ecotypes based on UPGMA method for ISSRs, grouped all ecotypes into five clusters (Figure 3). The first cluster (A), included two subgroups. The first subgroup (A1) consisted five seeded ecotypes; including one from Birjand, two seeded ecotypes of Behdan (w1, w2) and two black seeded ecotypes of Behdan (wb1, wb2). The second subgroup (A2) comprised seven ecotypes, including two seeded ecotypes of Chehardeh and Razg, four seeded ecotypes of Behdan (w5, w3, w4, w6) and one seedless ecotype of Noghab.

The second cluster (B) contained only two seedless ecotypes of Birjand and Behdan. In the third cluster (C), one seeded and one black seeded ecotypes from Behdan (w7, wb1) and one seedless ecotype from Nozad were located (Figure 3).

Table 6. Similarity matrix for the 20 barberry ecotypes investigated using SSR markers

Name genotype	w	W	w	c	wb1	w1	wb2	wb3	w2	w3	w4	w5	w6	C	w7	w8	c	c	c	c
1- w	1																			
2- w	0.28	1																		
3- w	0.38	0.48	1																	
4- c	0.25	0.40	0.34	1																
5- wb1	0.15	0.40	0.25	0.44	1															
6- w1	0.25	0.17	0.25	0.34	0.24	1														
7- wb2	0.14	0.25	0.23	0.40	0.35	0.32	1													
8- wb3	0.15	0.31	0.29	0.44	0.38	0.29	0.46	1												
9- w2	0.25	0.26	0.25	0.38	0.38	0.34	0.40	0.56	1											
10- w3	0.27	0.25	0.26	0.41	0.25	0.38	0.44	0.47	0.54	1										
11- w4	0.16	0.27	0.41	0.29	0.25	0.25	0.48	0.52	0.45	0.50	1									
12- w5	0.23	0.25	0.29	0.28	0.18	0.50	0.30	0.39	0.39	0.42	0.34	1								
13- w6	0.21	0.29	0.21	0.26	0.16	0.26	0.40	0.20	0.31	0.42	0.33	0.25	1							
14- c	0.20	0.14	0.16	0.30	0.36	0.47	0.33	0.25	0.30	0.21	0.20	0.44	0.09	1						
15- w7	0.22	0.29	0.33	0.22	0.32	0.16	0.29	0.22	0.26	0.19	0.33	0.31	0.08	0.35	1					
16- w8	0.22	0.25	0.35	0.16	0.12	0.35	0.20	0.07	0.12	0.18	0.17	0.33	0.23	0.22	0.13	1				
17- c	0.26	0.14	0.11	0.11	0.15	0.13	0.14	0.15	0.11	0.16	0.07	0.13	0.09	0.09	0.12	0.15	1			
18- c	0.18	0.21	0.19	0.18	0.33	0.28	0.36	0.23	0.33	0.36	0.29	0.27	0.25	0.23	0.20	0.26	0.23	1		
19- c	0.21	0.14	0.16	0.11	0.16	0.26	0.14	0.16	0.20	0.22	0.07	0.25	0.10	0.21	0.08	0.31	0.53	0.38	1	
20- c	0.28	0.18	0.15	0.14	0.09	0.18	0.08	0.09	0.14	0.15	0.04	0.17	0.06	0.20	0.16	0.23	0.20	0.25	0.21	1

In the cluster analysis based on SSRs, 20 ecotypes were grouped into six clusters (Figure 4). The first cluster (A), included four seeded ecotypes of Birjand, Chehardeh, Razg and Behdan (W7).

The second cluster (B), included two subgroups. The first subgroup (B1) consisted of three black seeded ecotypes from Behdan (Wb1, Wb2, Wb3), three seeded ecotypes from Behdan (W2, W3, W4) and one seedless ecotype from Birjand.

The second subgroup (B2), however, included two ecotypes from Behdan (W1, W5). Other two clusters (C and D) each consisted only one seeded ecotype from Behdan (W6 and W8), respectively.

The fifth cluster (E) further divided into two subgroups. Two seedless ecotypes of Noghab and Dorokhsh were located in the one subgroup (E1), while, the other subgroup (E2) consisted only one seedless ecotype of Nozad. The sixth group (F) had only one seedless ecotype of Afin (Figure 4).

The results of principal coordinate analysis, as a complementary method for cluster analysis, for ISSR and SSR markers were consistent with the results of cluster analysis.In this method, those ecotypes that are located in the same area of two- and three-dimensional plot supposed to have higher genetic similarity.

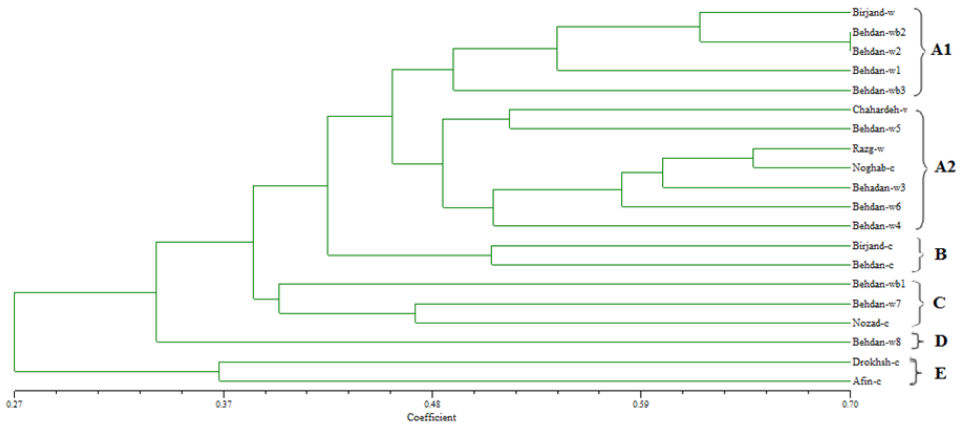

Figure 3. UPGMA clustering of barberry ecotypes based on Jaccard similarity coefficient calculated from ISSR markers

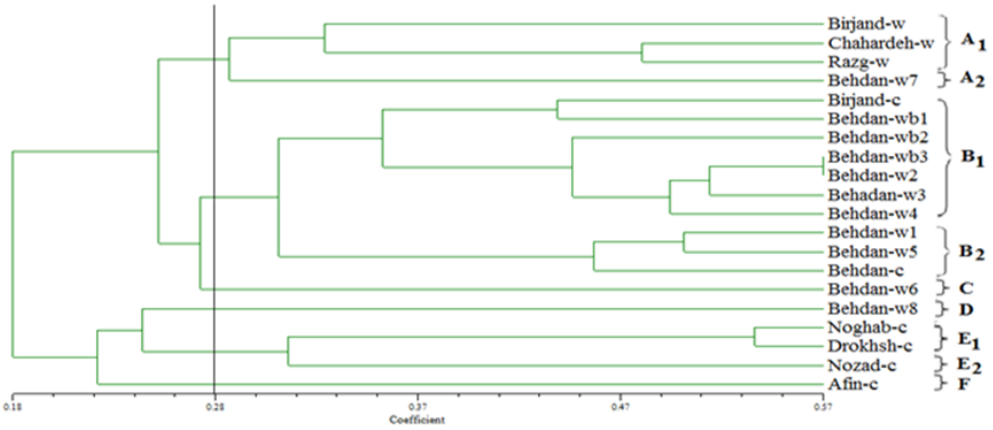

Figure 4. UPGMA clustering of barberry ecotypes based on Jaccard similarity coefficient calculated from SSR markers

Results of principal coordinate analysis based on ISSR and SSR markers were consistent with the cluster analysis result, in which those ecotypes hat were located in a group, came together in two- and three-dimensional distribution diagrams (Figures 5, 6).

Discussion

In this study, the genetic diversity of 20 barberry ecotypes from different regions of South Khorasan province, Iran was evaluated via SSR and ISSR markers. High levels of polymorphism obtained from ISSR and SSR markers representing a great diversity of the studied ecotypes as well as high power of the markers in the molecular diversity analysis.

The average PIC for ISSR marker systems was 0.65 representing the efficiency of this marker in differentiation of barberry populations. Analysis of the molecular variance showed that genetic diversity within populations was higher than between populations. According to the cluster analysis, classification of ecotypes did not follow the geographical diversity. With the closer look at the groups, most of ecotypes which located in a cluster had a higher similarity coefficient. In some cases, however, some ecotypes were grouped together despite the low level of similarity coefficient and high geographical distance, which it can be caused by differences in the quality of DNA affecting banding patterns, the lack of precision in scoring bands, non-uniform distribution of marker in the genome.

Also the results of the similarity matrix, PCoA analysis and cluster analysis showed barberry samples collected from the villages of Afin and Dorokhsh, had the low similarity and high genetic distance with other samples. The lowest percentage of similarity was observed between seedless barberry of Afin with seedless barberry of Birjand. Hybridization between populations with high genetic distance using genetic engineering techniques, protoplast fusion and in vitro fertilization can be an appropriate strategy for

improvement programs between populations of seedless barberry.

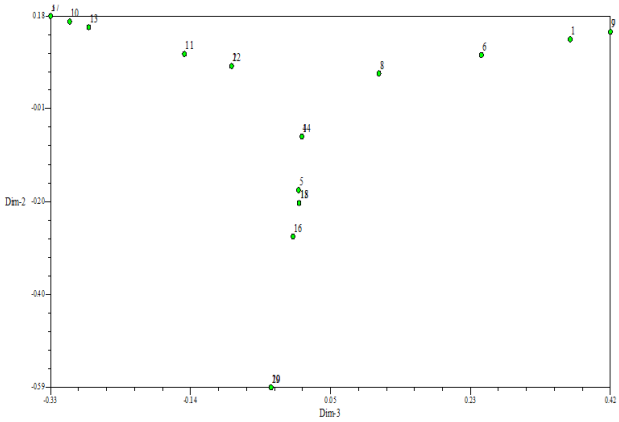

Figure 5. Distribution of the 20 barberry ecotypes revealed by PCoA analysis based on genetic similarity estimates calculated from ISSR data

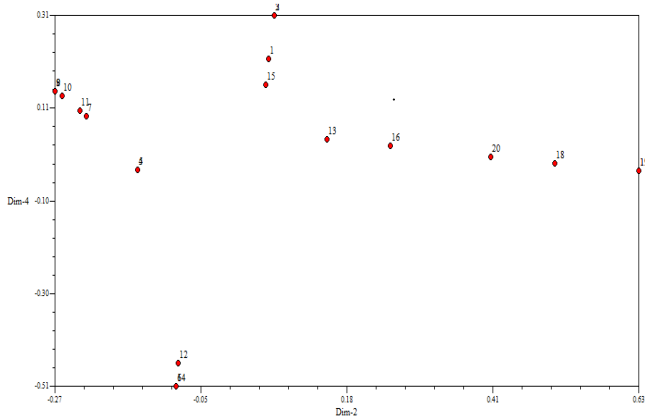

Figure 6. Distribution of the 20 barberry ecotypes revealed by PCoA analysis based on genetic similarity estimates calculated from SSR data

In a study by Bottini et al. (1999; 2000) on the genus Berberis native to Argentina, high morphological variation among Berberis species was observed and great differences in genomic content and ploidy levels were also reported.

Using the AFLP technique, Bottini et al. (2002) evaluated genetic variation of 13 Berberis species and the relationship within diploid and polyploid populations growing in southern Argentina and Chile. In general, results of the cluster analysis showed that populations of the same species were closely related in a group with high coefficients of similarity. In this study, collected ecotypes from South Khorasan province showed a high genetic diversity. All cultivated seedless barberry and wild barberry ecotypes collected from South Khorasan belong to B. integerrima (Rezaei et al., 2011). The seedless cultivars as B. integerrima were classified by both SSR and ISSR data within the wild type barberries. The high percentage of molecular variance within population indicating the genetic diversity between individuals of the same population. Rezaei et al (2011) assumed that self-incompatibility systems, which resulted from the occurrence of spontaneous mutations in B. integerrima, is responsible for the incidence of seedless barberry cultivars. They evaluated the genetic diversity of 45 barberry samples collected from Khorasan region using microsatellite markers. The results of cluster analysis and principal coordinate analysis showed that seedless barberry and species B. khorasanica had high affinity with B. integerrima species.

In agreement with the results of this study, Heidary et al. (2009) showed that the individuals of the seedless barberry cultivar were inserted between the population of B. integerrima based on AFLP markers. They studied the genetic diversity of 33 barberry ecotypes belonging to the 11 regions of Khorasan and two other species of ornamental barberry and one species of Mahonia aquifolium by AFLP marker and they concluded that barberry and Mahonia were located in two separate groups. At 17% similarity level, B. thunbergii was also located in a separate group from other species of the genus Berberis such as species of B. gagnepaini, B. vulgaris and B. integerrima which showed high level of similarities. Based on the results of this study, despite the geographical distances of samples of seedless barberry, they were in close genetic distance to each other.

Conclusion

Results from this study indicate that ISSR and SSR markers used in this study, showed high level of polymorphisms in this the genome studied ecotypes. High levels of polymorphism obtained from microsatellite markers representing a great diversity of the studied ecotypes as well as high power of this markers in molecular diversity analysis. High levels of PIC for ISSR57, ISSR1 and GA31 markers representing its high performance in the differentiation of used genotypes that we suggest them for similar studies. Characterization of the barberry germpalsms and understanding of genetic identity of cultivated and wild species can be used to improve quantity and quality of Berberis crops.

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Research Article

Molecular Detection of *Chlamydophila Abortus* In Aborted Fetal Tissues by Using Polymerase Chain Reaction (PCR) In Tabriz, Northwest of Iran

Mahsa Alem[1*], Reza Asadpour[2], Raziallah Jafari Joozani[2], Katayoon Nofouzi[1]

[1] *Department of Pathobiology, School of Veterinary Medicine, Tabriz University, Tabriz, Iran*
[2] *Department of Clinical Science, School of Veterinary Medicine, Tabriz University, Tabriz, Iran*

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Abstract

Enzootic abortion of ewes (EAE) induced by *Chlamydophila abortus* (formerly Chlamydia psittaci serotype1) is a major cause of reproductive failures in most sheep producing countries. In this study, the abortion prevalence of *Chlamydophila abortus* (*C. abortus*) in sheep abortion cases are evaluated by polymerase chain reaction (PCR) and objectives are considered Chlamydiosis incidence of abortion in sheep in the northwest region of Iran. For this purpose, the contents of fetal tissue from 50 aborted fetuses were homogenized and DNA extracted from them and then PCR is done using specific primers for Chlamydophila (CHOMP191, CHOMP371). The study showed that about 26% of the total sample is contained Chlamydophila. This study confirms that *C. abortus* as an important cause of ovine abortion in the northwest of Iran and showed the PCR in tissue pools of aborted fetuses is a useful method for rapid detection of *Chlamydophila abortus* Ovis infections.

***Keywords*:** *Chlamydophila abortus*, Molecular detection, Polymerase Chain Reaction (PCR)

Introduction

Ovine enzootic abortion (OEA) induced by *Chlamydophila abortus* (*C. abortus*) (formerly Chlamydia psittaci serotype 1) is a major cause of reproductive failures in most sheep producing countries (Aitken et al., 1993). In an epidemic of OEA, up to 30% of ewes abort in the last three weeks of gestation or give premature birth to weak or dead lambs. After the abortion, the ewes develop a protective immunity and, in endemically infected flocks, 5-10% of the ewes abort annually (Rodlakis et al., 1998; Aitken, 2000).

Despite the economically important losses due to late-term abortions and weak lambs, the prevalence of *C. abortus* is not well known in most Asian countries, including Iran. Diagnosis of OEA, by examination of fetal tissues following postmortem examination, can be achieved by a variety of methods.

All of these methods are based on direct antigen detection and include modified Ziehl-Neelsen (MZN) staining of placental smears and direct fluorescent-antibody staining of Chlamydiae in frozen cryostat sections of the placenta or fetal tissues. Several enzyme-linked immunosorbent assays (ELISAs) have been proposed for testing for *C. abortus* antibodies in sheep (Anderson et al., 1995; Longbottom et al., 2002).

However, the majority of sheep have preexisting background levels of antibody acquired from natural widespread infection by strains of C. pecorum. PCR appears to be an ideal alternate means of Chlamydiaceae detection because it offers advantages in sensitivity and reduced processing time over the conventional serological techniques. Furthermore, PCR does not rely on the presence and maintenance of viable organisms and poses less risk to laboratory staff than culture through eggs or cells. There have been many reports of PCR for the detection of Chlamydophila nucleic acids in specific animals or from particular tissues (Hewinson et al., 1997; Laroucau et al., 2001; Messmer et al., 1997; Bomhardvon et al., 2003), but at the time of the manuscript developing there are limited publications on the impact of a pool of samples in preparation for molecular detection of *Chlamydophila abortus* in aborted fetuses of ewes.

The aim of the present study was to determine the possible role of *Chlamydophila abortus* ovis as an etiological agent of ovine abortion by PCR in a pool of samples of varying tissues of aborted fetuses.

Corresponding authors E-mail:
**Mahsa.Alem@gmail.com*

Materials and Methods

Sampling Methods

During breeding season of 2011-2012, 50 abomasal aborted ovine fetus samples were collected from 10 flocks in Tabriz, northwest of Iran. Placentas were collected, macroscopically assessed for EAE lesions and representative cotyledons removed for molecular analysis. Following macroscopic examination, brain, skeletal muscle, liver, spleen, and abomasum were removed from each fetus. However, the condition of some of the fetuses was such that not all tissues could be collected. Fetal tissues and placental samples were then washed with PBS containing 1000 units/ml of penicillin (Pharmacia-Upjohn) and 1000 units/ml of streptomycin sulfate (Bristol-Myers Squibb) and then different tissues from each fetus were mixed in equal proportion and pulverized under liquid nitrogen. Approximately 200 mg of the sample powder stored as pool samples at −20°C until required for DNA extraction.

DNA Extraction of Tissues

Total DNA was extracted from approximately 200 mg powdered pool tissues using a commercial kit (Aquaprep DNA Tissue kit Bioneer, S. Korea) according to manufacturer's instructions. DNA concentration was measured at 260 and 280 nm (Biophotometer plus, Eppendorf, Germany). Electrophoresis of each DNA sample on 0.5% agarose gel in 1X Tris/Borate/EDTA (TBA) buffer was done to check the integrity of the DNA. A 60 µl aliquot of total DNA was produced from each sample and stored at -20 ºC until required for PCR analysis.

Polymerase Chain Reaction

DNA extract (5ul) was used to amplify a 630-bp fragment of ompA gene of Chlamydophila, using primers CHOMP191 (5′-GCI YTI TGG GAR TGY GGI TGY GCI AC-3′) and CHOMP371 (5′-TTA GAA ICK GAA TTG IGC RTT IAY GTG IGC IGC-3′), as described by Kaltenbock et al. (1997) and modified by Sachse and Hotzel (2005). One µl of the amplicon was used as template for the second-round amplification, using the primer set CHOMP218 (sense) (5′-GTA ATT TCI AGC CCA GCA CAA TTY GTG-3′)/ CHOMP336 (antisense) (5′-CCR CAA GMT TTT CTR GAY TTC AWY TTG TTR AT-3′), that yields a 400 bp product for *C. abortus*. Amplification reactions were carried out in a final volume of 25 µl, containing 100 ng of DNA, 0.5 µM of each primer, 2.5 µl 10X PCR buffer, 1.5 mM MgCl2, 0.2 mM dNTPs and 1 unit of Taq DNA polymerase. The following cycles were applied: initial denaturation step at 95ºC for 5 minutes followed by 35 cycles: denaturation at 94ºC for 30 seconds, primer annealing at 50ºC for 30 seconds, PCR products synthesis at 72ºC for 30 seconds and final synthesis step at 72ºC for 5 minutes. PCR products were recognized by electrophoresis on 1.5% agarose gel, stained with ethidium bromide and images were obtained in UVIdoc gel documentation systems (UK). Positive controls (DNA from references strains; ATCC 25116 and ATCC 2215), samples controls (DNA from abomasal fluids and lungs without etiological diagnostic), and none template control (distilled water) were included in each PCR run. PCR products with the molecular size of 440bp were considered indicative for identification as *C. abortus.*

Results

Genomic DNA was successfully extracted from aborted ovine fetus tissues using the DNA extraction kit. The PCR products of the primers specific for ompA gene (CTU-F and CTL-R) revealed the 630bp DNA fragment. Chlamydophila was isolated in 13 out of 50 cases of ovine abortion (26%). The positive control showed the excepted amplification product specific for Chlamydophila (630 bp) and *C. abortus* (440bp). PCR products obtained from these samples and *C. abortus* reference strains with the molecular length of 440bp (according to the new taxonomy: Chlamydia and Chlamydophila), are shown in Fig 1.

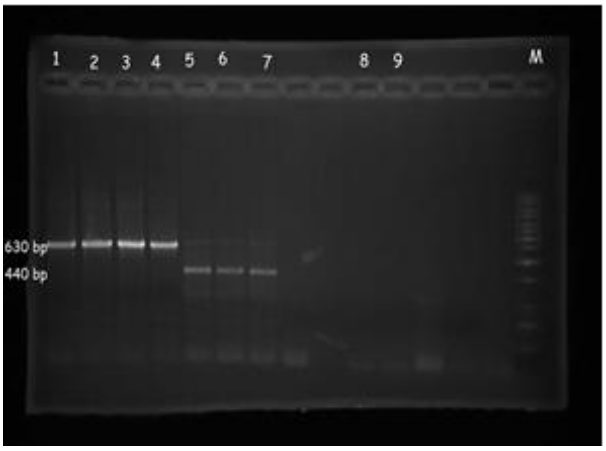

Figure 1. Ethidium bromide-stained 1.5% agarose gel electrophoresis of PCR products amplified using the mentioned primer. M, 100bp DNA ladder; lane 1, positive control sample of Chlamydophila; lane 2-4, Chlamydophila positive sample; lane 5, positive control sample of *C. abortus* ovis; lane 6-7, *C. abortus* ovis positive sample; lane 8, NTC (negative template control); lane 9, negative control (distilled water).

Discussion

OEA is one of the most important diseases affecting sheep flocks and because of abortion, weak lambs and a decrease in milk production can have negative economic effects. In previously reported serosurveys, the less-sensitive and -specific CFT had been used. Positive CFT titers can be caused by *C. abortus* as well as by C. pecorum.

According to the World Organisation for Animal Health (OIE) (2004), the most commonly used method for serodiagnosis of animal chlamydiosis the complement fixation test (CFT). However, the technique is laborious, has limited sensitivity and often impaired by cross-reactions between chlamydial species (McCauley et al., 2007). Recently developed serodiagnostic tests are mainly based on two main cross-reactive antigens present in all chlamydial species, lipopolysaccharide and the major outer membrane protein (MOMP) and thus, are not species-specific for diagnosing animals infected by OEA.

Other more specific tests need to be developed and evaluated in a European context, such as those based on specific monoclonal antibodies (Salti-Montesanto et al., 1997) and recombinant protein fragments (Sheehy et al., 1996). According to antigen detection, cultivation in cell culture is still regarded as the standard. While this time-consuming method is only applicable to cultivable strains, many strains are difficult to grow and not all laboratories have the facilities and expertise to culture. The possibilities for diagnostic detection of chlamydiae have considerably improved following the introduction of DNA-based methods, particularly the PCR, which permits direct identification from clinical specimens and differentiation of species. A number of tests have been published in the literature (Anderson et al., 1996; Kaltenbock et al., 1997; Messmer et al., 1997; Longbottom et al., 2001), but none of them has been validated in veterinary laboratories so far. One of the main difficulties in evaluating new tests for detection of *C. abortus* is a lack of a completely reliable method for comparative purposes.

Isolation, which was at one time used as the standard procedure has proved to have insufficient sensitivity (Wood et al., 1992; Domeika et al., 1994). Contamination of test samples with other bacteria, inadequate transport conditions, autolysis, antibiotic treatment, and other toxic factors may all interfere with the success of isolation (Sanderson et al., 1989). A further obstacle to isolation is the possible presence of chlamydiae in samples in the form of latent infection (the so-called “cryptic” form), which is well known in infections of the human uterine tube and joints (Sachse et al., 2005).

Methods based on nucleic acid and antigen detection are recommended for diagnostic uses. Some authors have found the former (Thiele et al., 1992) and others the latter (Domeika et al., 1994; Trevejo et al., 1999), to be more sensitive in the detection of Chlamydophila. In research by Lenzko et al. (2011), PCR and DNA microarray testing revealed the presence of chlamydiae in 78% of studied flocks. In sheep and goats, when *C. abortus* is present in the fetal membranes in large numbers, the two methods probably have almost identical sensitivity. In conclusion, our results showed that *C. abortus* is one of the important factors in abortions of sheep that are unknown and the vaccine has been applied to the control of abortions by this infection. Also, this study indicates that Chlamydia infection in the abomasal is an important factor for abortion in Iranian ovine.

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Research Article

Biological Activity of Persian Sturgeon Recombinant Growth Hormone Molecules Trapped In Inclusion Bodies (IBs)

Nasr Ehsan[1*], Hovhannisyan Hrachya[2], Pourkazemi Mohammad[1]

[1] *Iranian Fisheries Research Organization, P.O. Box: 14155-6116 Tehran, Iran*
[2] *Scientific and Production Center "Armbiotechnology" NAS RA, Yerevan, Armenia*

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Abstract

The biological activity of Persian sturgeon (PS) enfolded crude recombinant growth hormone (rGH) recovered from inclusion bodies (IB) estimated by administrating to fish fingerlings. The psrGH IBs expressed in *E. coli* were dissolved in cold guanidine HCl solution, immediately diluted ~1000 fold in cold sodium chloride 0.9% solution do not allow the rGH folding and administrated to fishes. It was revealed that intramuscularly injections of rGH at dosage of 0.5 µg/ g once a week for 8 weeks significantly accelerate the growth of fishes. Comparison of the mean growth rate and daily weight and length gain dates of Persian sturgeon fingerlings administrated by pure and crude grade rGH revealed the existence at list 2-5% potentially biologically active psrGH molecules in IBs. This fact enables to avoid the time and cost consuming processes of refolding and purification of rGHs.

***Keywords*:** Inclusion body, Persian sturgeon, Recombinant growth hormone, Biological activity

Introduction

The overexpression of recombinant proteins in industrially useful microorganisms showed, however, that recombinant protein did not form its native, soluble and bioactive conformation. Instead of native protein inactive aggregates (inclusion bodies) accumulated in the host cell (Rudolph and Lilie, 1996). Thus, the accumulated r-protein requires solubilization and folding steps prior to purification by chromatography, and the overall protein recovery is significantly affected by the efficiency of these pre-purification steps. Therefore, the accumulated proteins need to be solubilized using high concentrations of denaturants such as urea or guanidine hydrochloride (GnHCl), followed by removal of denaturants for protein refolding. Therefore, considerable efforts have been made to increase the efficiency of solubilization and refolding as a means of improving the overall recovery of biologically active r-protein from inclusion bodies.

However, expressing a protein in inclusion body form can be advantageous. Large amounts of highly enriched proteins can be expressed as inclusion bodies. Trapped in insoluble aggregates, these proteins are for the most part protected from proteolytic degradation (De Bernardez, 1998; Lilie et al., 1998; Misawa et al., 1999).

The major advantages associated with the formation of inclusion bodies are expression of a very high level of protein of the cellular, easy isolation of the inclusion bodies from cellular due to differences in their size, density and solubility as compared with cellular contaminants, lower degradation of the expressed protein, resistance to proteolytic attack by cellular protease and homogeneity of the protein of interest in inclusion bodies which help in reducing the number of purification step to obtain pure protein (Singh and Panda, 2005).

The general strategy used to recover active protein from inclusion bodies involves three steps: inclusion body isolation and washing; solubilization of the aggregated protein; and refolding of the solubilized protein. It is generally believed that recombinant therapeutic proteins to be properly folded and fully functional. However, recombinant proteins expressed in bacteria often are being made faster than they can fold into the native structure, accumulated in inclusion bodies (Marston, 1986; Schein, 1989). Although proteins trapped in insoluble inclusion bodies (IBs) are generally believed to be misfolded and inactive (Baneyx and Mujacic, 2004), some of current research no longer supports this assumption.A growing number of studies in the scientific literature describe IBs as

Corresponding authors E-mail:
* *Nasr_ehsan1357@yahoo.com*

entities formed by functional protein species with native secondary structure. Furthermore, the structural and functional diversity of the model proteins used in these studies leaves little room to speculate about these observations being artifacts or peculiarities of certain protein species (Garcia-Fruitos et al., 2007; Garcia-Fruitos et al., 2007
The aim of this study was evaluation of biological activity of psrGH from IB's by administration to fishes immediately after GnHCl solubilization without folding.

Materials and Methods

The recombinant *E. coli* DE3(rGH) was constructed earlier by our laboratory.

The psrGH butch fermentation in *E. coli*:

An aliquot (1 ml) of overnight Persian sturgeon recombinant Growth hormone (psrGH) *E. coli* DE3(rGH) overnight culture was inoculate in 10 ml of LB-broth containing 50 µg/ml ampicillin and grow at 37 °C by intensive aeration. After 2 hours of growth 100 µl IPTG was added to culture to induce rGH expression and incubation continued for 4 hours.

Inclusion body separation and rGH recovery

The cells harvested, suspended in 20 ml of 20 mM phosphate buffer (pH 7.2), and lysed by lyzocim. The resulting homogenate was centrifuged at 8000 rpm for 40 min at 4°C, and the pellet was washed with 20 m1 of 1 m sucrose and then with 20 ml of 4% Triton X-100, 20mM phosphate buffer, and 1 ml EDTA (pH 7.2) to remove soluble components, bacterial cell wall and cell membrane, and lipid components. The IBs were dissolved in cold guanidine HCl solution, diluted immediately ~1000 fold in cold sodium chloride 0.9% solution to minimize solvent concentration and do not allow the rGH refold and store in cold.

Fish rearing and rpsGH administration:

The fingerlings held indoors in pools (120 × 60 × 65 cm) filled with 400 l fresh water. Fresh water from a common reservoir supplied circularly, after filtration, at a running speed of 500-600 ml/min. Fish kept on a natural photoperiod at 23 - 26°C for 2 weeks before the study began. Each group of fish fed with commercial fish food pellets an amount of food equal to 3% of their total body weight twice daily. The IB suspension and purified hormone were intramuscularly injected to fishes once a week for 8 weeks.

The correlation index (CI)

CI was calculated by formula CI= weight/length

Statistical analyses:

Duncan's new multiple-range test (randomized block design). The 95% confidence level ($P < 0.05$) was used unless otherwise stated. Growth rates were compared using a one-way analysis of variance ANOVA (SPSS).

Results

The psrGH IBs dissolved in guanidine HCl solution and immediately diluted 1000 time in sodium chloride 0.9% solution to minimize Gnd HCl impact and not allow the rGH fold. A total of 60 PS fingerlings were randomly divided into four groups, with averages of 20±0.15 g for body weight and 17±0.2 cm for fork length and reared. One of the groups was control one next group was made intramuscularly injections of solubilized IB crude grade PS GH by dosage of 0.5 µg/g and the rest two groups were administrated of purified native rGH 0.01 and 0.05 µg/g dosages every week for 8 weeks. The morphometric characteristic (body weight and length) of fishes were recorded at the start of trial then every fourth week over 8-week period. The mean weight and length of fishes are presented in table 1.

Table1. Body weight and length of PS fingerlings injected by putre refolded rGH and crude grade solvents from IBs, over 8 weeks cultivation. (W- mean weight (g) and L - length (cm) of fishes. rGH IB – rGH from inclusive bodies, prGH – purified native rGH)

Cultivation period	Recombinant growth hormone dosage (*µg*/g)							
	Control		rGH IB 0.5		pnrGH 0.01		pnrGH 0.05	
	W	L	W	L	W	L	W	L
At the start	20.0	17.4	20.0	17.2	20.1	17.0	20.4	17.3
First 4 weeks	28.9	19.4	38.9	21.0	36.6	20.9	40.9	21.3
Second 4 weeks	35.1	20.7	44.7	22.5	42.2	22.3	49.0	23.0

Research Article

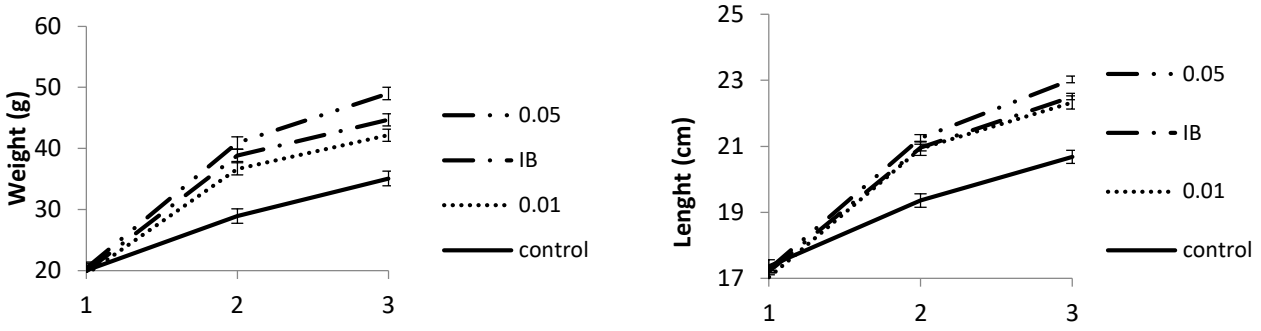

Figure 1. The influence of PS rGH administration on weight and length growth of fishes

At the end of 8th week, fish from groups received rGH and control group showed significant differences ($P < 0.01$) in weight and length (Table. 2). It was notable that growth enhancement of this treated groups were actually evident at the eight week Thus, after 8 weeks of rGH IB receiving group, the mean weight and length were 27.35% and 8.65% more than control group. Furthermore, the mean weight of groups 0.05 µg/g and 0.01 µg/g of pnrGH were respectively, 39.7% and 20.3% more than control group and the mean length of group groups 0.05 µg/g and 0.01 µg/g were respectively, 11.4% and 8.0% more than control group. At the end of experiment, the survivability of all fishes was 94 %. The daily weight increase of control fishes for 4 weeks was 144% while the increase weight of fishes administrated with dose 0.5 µg/g rGH IB, 0.01, 0.05µg/g pnrGH were 194.5%,188 % and 200% respectively but for the next 4 weeks were 115%, 115%, 120% and control was 121%. Length increase of control group for 4 weeks was 111% while the increase weight of groups administrated were 122%, 122%, 123% respectively but for the next 4 weeks were 107%, 107%, 108%, respectively, in groups administrated and control 106.7 %. Significant acceleration of fishes growth was observed for the first 4 weeks GH administration while the following administrations influence was no significant. It was revealed that once a week intramuscularly administrations of dissolved IB suspensions, were effective for first four weeks, after that the daily gains of treated and untreated fishes are not significantly differ (Fig. 1).

IB curve located between the curves of purified GH doses of 0.01 and 0.05 µg/g. As shown in Fig. 1 and 2 the first GH administration significantly increase growth rate of fishes for first 4 weeks but the 5th and following administrations were not show any influence on growth rate of fishes for the next 4 weeks comparing in control group.

Statistically analysis confirmed the trustworthiness of differences between the mean weight and length among group administrated with control group. Thus, one can therefore conclude that, unfolded IB solution accelerate the weight and length growth of Persian sturgeon. Due to the lack of appropriate standards in this bioassay, the activity of the psrGH IBs was quantified by extrapolation of the dates from growth curves of rGH administration.

The comparative studies of growth curves revealed that that the 0.5 µg/g dose of crude unfolded rGH solution possesses biological activity between 0.1 and 0.3 doses of purified and refolded hormone (Fig, 1).

Table 2. One-way analysis of variance ANOVA of the trial dates of weight and length.

ANOVA		Sum of Squares	Df	Mean Square	F	Sig.
Weight	Between Groups	22268.577	2	11134.288	291.861	.000
	Within Groups	7782.447	204	38.149		
	Total	30051.024	206			
Length	Between Groups	495.008	2	247.504	150.216	.000
	Within Groups	336.122	204	1.648		
	Total	831.130	206			

*Df- degrees of freedom; F- frequencies; Sig- signify

Thus IBs contain about 2-5% biologically active psrGH molecules or they are folded in fish body fluid. This finding enables to avoid the time and cost depending process of refolding and purification of rGHs from inclusion bodies.

Discussion:

Solubilization and folding of inclusion body proteins into bioactive forms is cumbersome, results in poor recovery and accounts for the major cost in production of recombinant proteins from prokaryotic organisms. Our results and other authors date shows that the yield GH is about 30% of total protein synthases in *E. coli*. During solubilization of IB, dialyse and purification a huge amount of recombinant molecules are lost. Because of, the previous purification GH is the main factor making its use more expensive and prohibitive to be used in aquaculture.

It was therefore necessary to develop more cost effective production of functionally active therapeutic proteins preparations for parenteral administration in crude grade form without the need for the proteins renaturation and purification. Many researchers describe IBs by functional protein species with native secondary structure (Garcia-Fruitos *et al*., 2007; Garcia-Fruitos *et al*., 2007; Jevsevar *et al*., 2005). Recent reviews in this area have reported IBs containing properly folded proteins (Doglia *et al*., 2008; Ventura and Villaverde, 2006), casting doubt on the paradigm of considering recombinant protein solubility as equivalent to protein conformational quality (Baneyx and Mujacic, 2004; Barannikova, 1987). However, none of them evaluated the possible effects of GH IBs crude preparations.

In this study for the first time was evaluate the effect of intramuscular administration of solubilized IB crude grade GH molecules to PS fingerlings and found significant increase in weight and in length of young fishes. From our date IBs demonstrated 20-30 fold less active than folded and purified GH, indicating on existence of 3-5% bioactive rGH proteins in intact inclusion bodies. Estimation of daily gain of weight and length revealed that once of week intramuscularly administrations of IB are effective during first four weeks, after that the daily gains are not significantly differ from control fihes.

Conclusion

For the first time demonstrated that crud IB hydrolysates 0.5 µg/g b.w. intramuscular administration accelerates weight and length growth of PS fingerlings equal with effectiveness of ~0.02 µg/g b.w. of purified rGH, indicating on the existence of about 2-5% bioactive recombinant GH molecules in IB aggregates.

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Review Article

Non-coding RNAs Could Be New Tools for Cancer Treatment

Atieh Teymoori[1], Mojtaba Teimoori[2], Madjid Momeni-Moghaddam[3*]

[1] *Department of Human Genetics, Golestan University of Medical Sciences, Gorgan, Iran*
[2] *Urology Research Center, Razi Hospital Rasht, Guilan, Iran*
[3] *Department of Biology, Faculty of sciences, Hakim sabzevari University, Sabzevar, Iran*

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Abstract

For 50 years, the term gene is synonymous with regions of the genome gene that coding by mRNAs and translate to protein. nonetheless, Genome wide Recent studies have revealed that regulating gene expression through degradation or translational inhibition of their point mRNAs and thus attend in a wide variety of physiological and pathological cellular processes including: development, cell proliferation, differentiation, and apoptosis pathways by thousands of regulatory non coding RNA such as lncRNAs and microRNAs. According to a recent survey, it is known this RNAs have vital role in regulation cellular pathways at transcriptional, posttranscriptional and epigenetic levels. These noncoding genes are often aberrantly expressed in a variety of human cancers. However, the biological functions of most ncRNAs remain largely in doubt. In this review, we proved that a remarkable part of the genetic etiology of cancer is imposed by noncoding regulatory sequences. The purpose of this review is aimed to give an outlook of using of noncoding RNA as diagnostic markers and therapeutic targets. These observations emphasized that the recognition of coding genes and Research continued evolution and function of non-coding RNAs for a comprehensive understanding human complex diseases like cancer are essential.

***Keywords*:** NcRNA, Expression, Transcription, Cell proliferation, Apoptosis, Cancer

Introduction

Cumulative evidence supports the importance of changes in the steps following the transcription of gene expression associated with cancer symptoms. The steps following transcription (i.e. pre-mRNA splicing and polyadenylation, with 'pre-mRNA' connoting the immature mRNA), stability and translation of mRNA (changes in mRNAs) and post transcriptional regulators (RNA-binding proteins and noncoding RNAs such as microRNAs and long non-coding RNAs) are very diverse and constantly growing. Understanding this diversity is therefore challenging for cancer treatment.

Noncoding RNAs are a diverse family of regulatory transcripts that are effective in all the steps of gene expression, from transcription and mRNA stability to mRNA translation. Recent evidence has revealed the critical role of noncoding RNAs in the pathogenesis of cancer.

Large-scale cDNA sequencing projects along with technological advances such as tiling arrays and new generation RNA sequencing have provided a new perspective on the complexity of transcriptome, i.e. a set of mRNA molecules or transcripts expressed in a cell (Consortium, 2012). While a number of protein-coding genes (20,000-25,000) have retained their broad consensus, recent human transcriptome studies have uncovered a significant number of non-coding RNAs (ncRNA). These transcribed elements lack the capacity to encode a protein and are confusingly abundant in all the organisms studied to date, from yeast to humans (Birney et al., 2007; Kapranov et al., 2007) .These non-coding portions of the genome produce a wide variety of mostly regulatory RNAs that often differ in terms of biogenesis, properties and function and are divided into short RNAs, such as microRNAs (Lin and Gregory, 2015), and long RNAs (>200 nt), depending on their size (Carninci et al., 2005; Guttman et al., 2009).

Extensive studies have been conducted to examine the role of ncRNAs in cell biology and cumulative evidence shows that these RNA molecules play significant roles in cellular functions and their restructuring leads to various severe pathological conditions, including cancer (Calin and Croce, 2006; Tsai et al., 2011).

Corresponding authors E-mail:
* *iranbioman@yahoo.com*

Preliminary evidence suggests that ncRNAs, especially long ncRNAs (lncRNAs), have a key role in tumor development (Huarte and Rinn, 2010), and that lncRNA-mediated biology has a major role in cancer progression(Prensner et al., 2011).LncRNAs are classified into several broad categories by their mechanisms of the regulation of mRNA transcription and translation (Fatemi et al., 2014)or LncRNAs can regulate apoptosis and cell cycle(Kino et al., 2010), or, as positive regulators of gene expression, increase the expression of neighboring genes (Andersson et al., 2014), LncRNAs play a significant role in epigenetic regulation, and acting as a modular scaffold, they assemble protein complexes to position epigenetic enzymes to the specific sequences (Guttman et al., 2011; Khalil et al., 2009). Some of important LncRNAs involved in cancer listed in table-1.

Approaches to Cancer Treatment

Targeted cancer therapies are medications or other substances that prevent the growth and spread of cancer by interfering with specific molecules (molecular targets) that are involved in the growth, development and spread of cancer.

Many different targeted therapies have been approved for use in the treatment of cancer, including hormone therapy, signal transduction inhibitors, regulators of gene expression, apoptosis inducers (programmed cell death), angiogenesis inhibitors, immunotherapy (to boost the immune system to attack tumor genetic mutations) and toxin delivery molecules (delivery of toxin into cancer cells).

Hormone therapy slows or stops the growth of hormone-sensitive tumors that depend on certain hormones for their growth. Hormone therapy helps treat cancer by blocking the production of the hormones in the body or by interfering with their action. Hormone therapy is useful in the treatment of breast and prostate cancer (Khalil et al., 2009; Sweeney et al., 2015).

Signal transduction inhibitors block the activities of the molecules involved in signal transmission –a process by which cells respond to the signals received from the environment. During this process, when the cell receives a certain signal, the signal is relayed within the cell through a series of biochemical reactions and ultimately leads to appropriate response(s). In some cancers, malignant cells are stimulated, but not by external growth factors and are constantly divided. Signal transduction inhibitors disrupt this improper signaling (Steeg, 2003). Regulators of gene expression modify the function of proteins involved in controlling gene expression.

Apoptosis inducers subject cancer cells to a process of controlled cell death called apoptosis. Apoptosis is a method of cleansing the body of unneeded or abnormal cells, but cancer cells use strategies to evade apoptosis, i.e. cellular processes that cause genetic and physiological changes in them. Apoptosis inducers cause the death of cancer cells by circumventing these strategies (Hassan et al., 2014).

Angiogenesis inhibitors block the growth of new blood vessels into the tumor (a process called tumor angiogenesis). Tumors need a blood supply to grow beyond a certain limit, as blood provides the oxygen and nutrients needed for the continued growth of tumors. Treatments that prevent angiogenesis may thus stop tumor growth as well. Some targeted therapies that inhibit angiogenesis interfere with the function of vascular endothelial growth factor (VEGF), which is a substance that stimulates the formation of new blood vessels. Other angiogenesis inhibitors target other molecules that stimulate the growth of new blood vessels (El-Kenawi and El-Remessy, 2013).

Immunotherapy is a method of treatment that destroys cancer cells by triggering the immune system. Some types of immunotherapy consist of monoclonal antibodies that identify specific molecules on the surface of cancer cells. Monoclonal antibody binding to the target molecule leads to the immune destruction of cells that express the target molecule. Other monoclonal antibodies bind to certain immune cells to help them kill more cancer cells. (Kyi and Postow, 2014). Monoclonal antibodies that deliver toxic molecules can cause the death of cancer cells in a certain way. When the antibody binds to its target cell, the toxic molecule that is bound to the antibody (for instance, a radioactive substance or toxic chemicals) is absorbed into the target cell and eventually causes cell death. The toxin will not affect cells that lack a target for the antibody; that is, it does not affect the healthy cells and seeks only the target cells (for instance, it does not affect the vast majority of the cells in the body).

Cancer vaccines and gene therapy are sometimes considered targeted therapies, as they have a special role in the growth of cancer cells. More information about these therapies can be obtained through NCI fact sheets on cancer vaccines and biological therapies for cancer (Imai and Takaoka, 2006).

Traditional Treatments

Including surgery, radiotherapy and chemotherapy, either alone or in combination with other methods, are the most commonly used

methods used for the treatment of cancer. The method of treatment used differs depending on the type of cancer, the extent of the disease, its rate of progression, the patient's conditions and the response to the treatment.

Surgery

Although the development of other therapeutic strategies has reduced the rate of surgical intervention in the treatment of certain cancers, surgery is still the oldest and principal form of cancer treatment. Despite the advances in surgical techniques, the capacity of surgery to control cancer is limited by the fact that, at the time of surgical intervention, two-thirds of cancer patients have tumors that have spread beyond the original site.

Radiotherapy

In this method, cells get destroyed by radiation for two reasons: Either because they are no longer able to proliferate as a result of excessive genetic damage or because radiation induces apoptosis or programmed cell death. Cancer cells are more sensitive to radiation compared to healthy cells, since they are constantly proliferating; this greater rate of proliferation makes cancer cells weaker than healthy cells, which are not always proliferating, and as a result, cancer cells are less able to recover from radiation damage. Radiation therapy is the most effective method for eradicating an undetectable disease at the periphery of the tumor and the least effective method for killing cells at the center of a large tumor. In general, 'chemotherapy' refers to the use of chemical compounds or medications for eliminating diseases; nevertheless, the term is often exclusively used for cancer and interchangeably with anticancer agents. Chemical compounds developed for chemotherapy destroy cancer cells by preventing their proliferation. Unlike surgery or radiotherapy, which often fail to treat widespread metastasis, medications can spread throughout the body through the bloodstream and attack the tumor cells growing anywhere, except for a few places in the body that are known as sanctuary sites, i.e. areas in which medications cannot access the cancer cells (Tannock, 1998).Research into lncRNAs in cancer and the identification of a number of lncRNAs (long non-coding ribonucleic acids) have led to the generation of new hypotheses about the biology of cancer cells. The present study reviews the current perceptions of ncRNAs in cancer with a special emphasis on lncRNAs as new triggers of angiogenesis. The present review focuses on the general features of lncRNA, their mechanisms of action and their role in the development of cancer.

Non- coding RNAs Gene Therapy

LncRNAs have an advantage over protein coding genes as potential biomarkers and therapeutic targets, as their gene expression is more tissue specific, which makes them attractive as a biomarker and therapeutic target. LncRNAs are remarkably stable in body fluids and tissues; they are also valuable biomarkers in liquid biopsies and facilitate the inhibition of invasive procedures (Qi and Du, 2013; Tong and Lo, 2006). LncRNAs can be used with therapeutic targets in a variety of methods, including RNAi mediated gene silencing, antisense oligonucleotides, targeted plasmid, small molecule inhibitors and gene therapy, as discussed below (Sánchez and Huarte, 2013; Takahashi and Carninci, 2014). Evidently, lncRNAs are crucial to the epigenetic control of gene expression and comprise potential therapeutic targets for conventional antisense technologies. In particular, in cases where a lncRNA is directly linked to the pathogenesis of the disease, conventional RNAi or antisense oligonucleotides can be used for regulating gene expression.

The Hallmarks of Cancer

Hanahan and Weinberg (2000) described six properties required for cell transformation, coined as the hallmarks of cancer. These properties include self-sufficiency in growth signals, insensitivity to antigrowth signals, evading apoptosis, limitless replicative potential, sustained angiogenesis and tissue invasion and metastasis (HANAHAN AND WEINBERG, 2000). LncRNAs are regulatory molecules that are involved in most of these functions and key patterns thus emerge (Gutscgner and Diederichs, 2012).

Self-sufficiency In Growth Signals

LncRNAs often increase self-sufficiency in growth signals by activating the signal receptors in the first step of signal transduction. Multiple lncRNAs specifically bind nuclear receptors either alone or in a ribonucleoprotein complex (CATHCART ET AL., 2015). LncRNAs often induce self-sufficiency in growth signals by activating the signal receptors in the first step of the signal transduction. Some lncRNAs, such as PVT1, affect cell proliferation by regulating receptor abundance, as previously shown for PVT1 and thyroid-stimulating hormone (Zhou et al., 2016).

Insensitivity to Antigrowth Signals

Inhibiting or evading growth can also be regulated by lncRNAs –a process that is often carried out by the effect of RNA on tumor suppressors that regulate

cell cycles such as cyclins, CDKs, CDK inhibitors and p53(KITAGAWA ET AL., 2013). PANDA suppresses protein CDKN1A through PRC1 while ANRIL (a type of RNA) suppresses target tumor suppressor protein p15 (CDKN2B) through PRC2 (Kotake et al., 2011; Puvvula et al., 2014). Some lncRNAs regulate the expression of tumor suppressors by affecting different parts of transcription and translation. Transcription initiation can be affected by the scaffolding of transcription factor complexes, as in the case of LincRNA-p21 and p21 (CDK2 inhibitor) (DIMITROVA ET AL., 2014).

Evading Apoptosis

Apoptosis or controlled cell death is one of the key pathways for the control of carcinogenesis (ROSSI AND ANTONANGELI, 2014). Some lncRNAs act in the regulation of transcription of key apoptotic genes. For example, lncRNA INXS is expressed from the intron of Bcl-X and regulates its splicing into a pro-apoptotic isoform inhibitor of apoptosis (DEOCESANO-PEREIRA ET AL., 2014).

Sustained Angiogenesis

Multiple lncRNAs are mainly involved in the regulation of nutrient supply to the tumor by regulating the VEGF, which is essential for the formation of blood vesicles. According to recent reports, the transcription of VEGF is regulated by lncRNAs HOTAIR (Fu et al., 2016).

Tissue Invasion and Metastasis

Multiple lncRNAs increase the invasiveness of cancer cells and facilitate metastasis. Examples include the RNAs h19 and MALAT1 in colorectal and nasopharyngeal carcinoma (Raveh et al., 2015; Yang et al., 2015) .

LncRNAs in Cancer

This section discusses a number of important deregulated lncRNAs in cancer and their mechanisms of action and potential clinical applications.

KCNQ1OT1 (KCNQ1 Overlapping Transcript 1) is another imprinted, paternally expressed 91.5 kb transcript produced from the KCNQ1 locus, a few hundred kilobases away from H19 (Mohammad et al., 2008), that regulates gene expression epigenetically by interacting with chromatin remodeling complexes like PRC1, PRC2 and G9a proteins for silencing KCNQ1 (Nakano et al., 2006; Pandey et al., 2008). It is a CRISPR RNA and the chromosomal aberrations (any general changes in the chromosome structure is called aberration) associated with it include Beckwith-Wiedemann syndrome, which is a congenital overgrowth syndrome (Higashimoto et al., 2006; Weksberg et al., 2002), colorectal cancer (Nakano et al., 2006) hepatocellular carcinoma (Wan et al., 2013) and pediatric adrenocortical tumors (Wijnen et al., 2012).

NEAT1 (Nuclear Enriched Abundant Transcript 1) is a gene that produces two transcripts: the 37 kb NEAT-1-1 short isoform and the 32 kb NEAT-1-2 long isoform. Although the expression of the long isoform is much lower compared to the short isoform, NEAT1 is widely expressed across several tissues. NEAT1 is found exclusively in the paraspeckles (dynamic nuclear structures) in the nucleus (Naganuma and Hirose, 2013; Sunwoo et al., 2009) and plays an important role in the regulation of gene expression in transcription and after transcription, and its reduced expression leads to the disintegration of paraspeckles (Clemson et al., 2009). In fact, NEAT1 and NEAT2 (MALAT1) transcription shows that their model of binding to the human genome depends on hundreds of active genes. NEAT1 is strongly induced in breast cancer cells and is also involved in the transformation of myeloid cells into acute promyelocytic leukemia or APL (Zeng et al., 2014). In addition, its positive over-regulation in ATRA (All Trans Retinoic Acid) induces the differentiation of NB4 (APL) cells that could be inhibited by specific siRNA for NEAT1 (Zeng et al., 2014). Silenced NEAT1 in Burkitt's lymphoma cells leads to a reduced viability, increased apoptosis and therefore an abnormal cell morphology, thereby suggesting their oncogenic nature (Halford, 2013).

GAS5 (Growth Arrest Specific 5) at 1q25.1 locus produces two splice variant lncRNAs and its intron also leads to the formation of several snoRNAs (Mourtada-Maarabouni et al., 2008). GAS5 acts as a tumor suppressor and facilitates normal growth inhibition and apoptosis through the repression of GR (glucocorticoid receptor) mediated transcription (Pickard and Williams, 2014). GAS5 interacts specifically with the DNA binding domain of GR and inhibits the binding of GR to its target genes, including cIAP2 (cellular Inhibitor of Apoptosis 2), bringing about apoptosis, independent of other triggers in cancer cells. GAS5 also represses progesterone receptor and androgen receptor in a ligand-dependent method (Mourtada-Maarabouni et al., 2008). It also induces the inhibition of mTOR (mammalian Target of Rapamycin), which regulates protein synthesis and cell growth and proliferation. Observations have proved the fact that the

antiproliferative effect induced by Rapamycin can be repressed by silencing GAS5 in primary T cells as well as in the leukemic cells (Mourtada-Maarabouni et al., 2010).In turn, GAS5 is regulated by a negative feedback loop with miR-21(Zhang et al., 2013) .The down-regulation of GAS5 and/or its snoRNAs along with genetic aberrations at the locus (chromosomal locus) are associated with mild carcinogenesis in several cancers, including breast cancer (Mourtada-Maarabouni et al., 2009).

HULC (Highly Up-regulated in Liver Cancer), size 1.6 kb, is transcribed from the 6p23.3 locus (Panzitt et al., 2007) reached this finding with the help of Hepato Cellular Carcinoma (HCC) specific microarrays as the most highly up-regulated lncRNA in this cancer. Just as a typical mRNA, it has two exons and a poly A tail and is strongly localized in the cytoplasm and cooperates with ribosomes in the cleansing process but does not encode for any protein. It separates miRNAs and is involved in inhibiting the suppression of miRNAs that induce repression. Liu et al. (Liu et al., 2012) reported that the SNP, rs7763881, in HULC, is significantly associated with HCC susceptibility in HBV (Hepatitis B Virus) carriers. In addition, the reduced expression of CREB (cAMP response element-binding protein) and the use of a PKA (Protein kinase A) inhibitor reduces the regulation of HULC, showing that phospho CREB is required to activate HULC (Wang et al., 2010). HULC is oncogenic in nature and is highly up-regulated in both tumors and the plasma of HCC patients, but it has never been detected in any other tissues or cancers related to them (Panzitt et al., 2007). It, therefore, acts as a specific non-invasive biomarker for HCC (Xie et al., 2013). In addition, it is not expressed in primary colorectal cancers, but is detected in colorectal cancers metastasizing to the liver and associated with specific cancer symptoms for the hepatic tissue. Highly Up-regulated in Liver Cancer (HULC) is a definite symptom of hepatic cancer (Matouk et al., 2009). LncRNAs bind to miRNA-binding regions to separate the miRNAs and thus regulate the activity of miRNAs (Wang et al., 2010).

PCAT1 (Prostate Cancer Associated ncRNA Transcript 1) is a 7.8 kb lncRNA transcribed from the 8q24.13 locus.This RNA is up-regulated in metastatic cancers and high grade prostate tumors. Prensner et al. (Prensner et al., 2011) identified 121 prostate cancers associated with PCATs by RNA sequencing analysis from prostate cancer tissues in which PCAT1 is highly up-regulated. The reduced expression of PCAT1 in androgen dependent prostate cancer cell line leads to the alteration of hundreds of genes (Prensner et al., 2011). PCAT1 has also been reported to play an important role in double strand DNA break repair and to inhibit the homologous recombination of DNA (Prensner et al., 2014). It is a transcriptional repressor of DNA repair genes, just as BRCA2 tumor suppressor, and is instead regulated by PRC2. The overexpression of PCAT1 is associated with increased sensitivity to PARP inhibitors due to the reduction in RAD51 foci formation. PCAT1 is a negative prognostic marker for prostate cancer (Prensner et al., 2011). These prostate specific lncRNAs appear to be very useful in the process of treatment as diagnostic and prognostic markers in prostate cancer because traditional markers such as PSA have only a limited prognostic value. Several lncRNAs contribute to the regulation of p53 tumor suppressor signaling (Pickl et al., 2014). MEG3, a maternally expressed imprinted lncRNA on Chr14q32 activates p53 and facilitates p53 signaling, including the enhancement of p53 binding to target gene promoters (Zhou et al., 2007). MEG3 binds to p53 signals in meningioma and suppresses MEG3 overexpression, cell proliferation in meningioma and hepatocellular carcinoma cell lines (Braconi et al., 2011; Zhang et al., 2010). In human tumors,asignificant reduction in MEG3 expression is observed with the frequent hypermethylation of its promoters in pituitary tumors (Gibb et al., 2011) and leukemias(Benetatos et al., 2010). Overall, these findings suggest that MEG3 is a tumor suppressor. MEG3 is a modified lncRNA gene expressed in the maternal allele. The modification of this gene is induced through the binding of cytosine to methylation controlling binding proteins such as CTCF (Rosa et al., 2005). MEG3 is silent in many cancer cells due to DNA methylation (Benetatos et al., 2011; Zhao et al., 2005). MiR-29 and miR-148 can regulate DNA methyltransferase (DNMT) 1 and 3 by increasing the expression of MEG3 in hepatocellular cancer and gastric cancer, respectively (Braconi et al., 2011; Yan et al., 2014). MEG3 is a relatively poor prognosis in gastric cancer, pituitary adenomas, tongue squamous cell carcinoma and lung cancer (Lu et al., 2013; Sun et al., 2014). Yin et al. found that the low expression of MEG3 is associated significantly with low histological grade (proximity of the tumor to the main tissues) and deep tumor invasion in colorectal cancer (Yin et al., 2015). However, the metastasis mechanism of cancer cells MEG3 is not very clear. Examinations showed that MEG3 may suppress tumor proliferation through p53- dependent and/or p53-independent pathways (Lu et al., 2013; Zhou et al., 2007).

Table 1. LncRNA involved in cancer.

LncRNA	Genomic location	Official Full Name	Expression in patients or cancer cells	Function in tumorigenesis
KCNQ1OT1	11p15	KCNQ1 Opposite Strand/Antisense Transcript 1	Increased expression in colorectal cancer(Nakano et al., 2006)	NA
NEAT1	11q13.1	nuclear paraspeckle assembly transcript 1	Down-regulated in acute promyelocytic leukemia cells(Zeng et al., 2014)/ increased expression in breast cancer cell lines (Choudhry et al., 2015)	Oncogene
GAS5	1q25.1	Noncoding RNA growth-arrest-specific transcript 5	Down-regulated in breast cancer (Mourtada-Maarabouni et al., 2009)	Tumor suppressor
HULC	6p24.3	Highly up-regulated in liver cancer	Increased in HCC and colorectal cancer liver metastasis)Wang et al., 2010) / (Liu et al., 2012)	Oncogene
PCAT1	8q24	Prostate cancer associated transcript 1	Increased in a subset of prostate cancers(Prensner et al., 2011)	Oncogene
MEG3	14q32.2	Maternally expressed gene 3	Down-regulated in multiple cancers)Benetatos et al., 2011)	Tumor suppressor
ANRIL	9p21.3	Antisense NcRNA in the INK4 Locus (CDKN2B antisense RNA 1)	Inversely relates to p15 expression in cancer(Kotake et al., 2011)/ (Yap et al., 2010)	Oncogene

ANRIL (Antisense Noncoding RNA at INK4 Locus), also known as p15AS, is an antisense transcript of CDKN2B at 9p21.3 locus that has several alternatively spliced isoforms, including 3.9 kb and 34.8 kb transcripts(Kotake et al., 2011) (Yu et al., 2008). The mis expression of ANRIL is associated with a variety of diseases, including cancer.(Iacobucci et al., 2011; Popov and Gil, 2010). ANRIL creates changes in gene expression through epigenetic methods as it binds to PRC1 and PRC2 and induces gene silencing at the INK4b-ARF-INK4a locus (Kotake et al., 2011). It binds specifically to SUZ12 (Suppressor of Zeste 12 homolog), a subunit of PRC2, and induces the repression of p15, a tumor suppressor gene; as a result, the inhibition of ANRIL induces p15 and reduces cell proliferation (Kotake et al., 2011).
Nevertheless, these data are obtained from studies conducted on different cell types and it is not clear whether ANRIL binds to both complexes simultaneously or not. In addition, ANRIL has a highly complex splicing pattern with numerous variants, including circular RNA isoforms, and its expression has been detected in many tissues.

Conclusion

Any research involved in cancer treatment and prevention is very important due to worldwide cancer problems and among of new techniques non-coding RNAs are so important because the can affect very specific. Nowadays these small molecules presented as targeted tools for cancer therapy so knowing any mechanism about them are so important for researcher, in this review we mentioned some critical issues about them including a brief introduction and describe they role in cancer, treatment and prevention by reviewing some good related articles. We believe these small molecules will be work as a big and potent tools in cancer treatment and will play their clinical roles very soon.

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