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Table of Contents

Significance of Cell/Stem Cell Therapy in Wound Care Management

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Summary

Wound care management is a continuous challenging task for researchers and tissue engineers. Skin substitutes (synthetic and natural) have been introduced as emergency replacements/grafts to the damaged skin and a number of problems such as infection, graft rejection, inadequate healing, short shelf life etc. have reduced their clinical importance as being the ideal skin substitutes. A number of novel ideas have been presented in last decades which have focused on the applications of stem cells as ideal candidate in the development of ideal skin substitutes.

Keywords: Cell Therapy, Stem Cell Therapy, Wound Healing, Wound Management

Introduction

Skin grafting processes starting from 1871 by Reverdin, up till now has been considered as a challenging task for researchers and tissue engineers and a number of skin substitutes, containing degradable synthetic or biological components have been introduced and are being considered as emergency replacements/grafts to the damaged skin for example, Biobrane®, Integra®, OrCel®, Suprathel® etc are available for clinical utilization(Irfan-Maqsood and Hemmati Sadeghi, 2013). There are a number of post grafting problems including infection, graft rejection, inadequate healing, short shelf life etc. associated with currently available skin substitutes. This necessitates the need for development of innovative tissue engineering approaches based on biological scaffolds and clinical grade stem cells could be an attractive alternative for available skin substitutes.Reliable and xenobiotic-free keratinocyte culture techniques(Hannigan et al., 1996), better understanding of the molecular mechanisms in the regulation of epidermal stem cells (Li et al., 2007), techniques to accelerate basement membrane formation and vascularization, solution to post grafting problems associated in skin engineering, such as graft contraction, loss of pigmentation and scars formation (Islam and Zhou, 2007; Li et al., 2013; Thiery, 2003) are suggested as main priorities in the field. Graft necrosis,

extensive inflammatory reaction, marked foreign-body reaction (FBR), rapid scaffold degradation, abnormal collagen deposition and remodelling still remain the major issues in skin bioengineering (Nakamura and Tokura, 2011; Yan et al., 2010). Problemsassociated with chemical scaffolds, perceive the ideas of biological membranes as alternatives (Mohd Hilmi et al., 2013). Application of stem cells, especially mesenchymal stem cells, along with keratinocytes, and identification of specific antigens for keratinocyte grafts would serve as promising elements in skin bioengineering.

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Open Access Statement:

Identification of PI3K Isoforms in Human Prostate Cancer Cell Lines (PC3, DU145) and Human Bladder Carcinoma Cell line (5637)

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Abstract

There exists an association between PI3K pathway licentious activity and the considerable feature of high metastatic potential of the genitourinary cancer cells. Although DU 145 and 5637 have functional phosphatase and tensin homolog (PTEN) tumor suppressor gene, which antagonizes PI3K function, PC-3 is null for PTEN gene. In pursuit to explain why PTEN bearing cell lines display high metastatic behavior, we searched for any discrepancy in PI3K isoforms expression pattern between these cell lines. Gathering gene bank data files, specific primers were designed, for all the genes of 12 studied isoforms from 3 different classes of PI3K. Total RNA was extracted and examined by Real- Time PCR to compare the cells for the type and amount of the isoforms which expressed. Cα and R2 isoforms are indicative of an equal expression for PC3 and DU145, R3 transcripts revealed 80% decrease in DU145 and Cβ, R1 and C2α demonstrated an increased expression in DU145. When a comparison is made between 5637 and PC3, it can be seen that although a little decrease in the level of R3 transcripts was demonstrated, the amount of Cα, Cβ, R2, R1 and C2α increased. In conclusion in this study it is proposed that R1, R2, Cα, Cβ , C2α and R1, Cβ , C2α are candidate genes for silencing via RNAi in 5637 and DU145, respectively, to evaluate their roles in metastatic behavior of the both studied PTEN bearing cell lines.

Keywords: PI3K Isoforms, Prostate, Bladder, 5637, DU145, PC3

Introduction

Phosphatidylinositol 3-kinases (PI3Ks) as lipid kinases devise pivotal roles in regulation of DNA repair, apoptosis, cell cycle, angiogenesis, metabolism and cell motility. They act as intermediate signaling molecules and transmit signals from cell surface to the cytoplasm (Akinleye, 2013). PI3K has been reported to have protein kinase activity as well. However, it remains unclear if this protein kinase activity has any role in vivo. (Vanhaesebroeck, 2012; Cantrell, 2001; Vanhaesebroeck and Waterfield, 1999).

There are at least twelve members of this family present in the human genome (Fry, 2001). There are eight mammalian PI3K enzymes that have been divided into three classes according to structural features and lipid substrate preferences (Vanhaesebroeck, 2010; Fruman, 2014; Kastan and Lim, 2000). In mammals, class I PI3Ks are the best understood and are present in all cell types (Fruman, 2014).

Only class I PI3Ks are involved in cancer. Based on this fact it can be deducted that the three classes of PI3K have different product and substrate specificities. Only class I PI3Ks can use PIP2 to generate PIP3, class II PI3Ks produce the 3,4-bisphosphate and the 3-monophosphate of inositol lipids, and class III can only make the 3-monophosphate(Zhao , 2008).

It should be noted that Class I enzymes are able to convert PI (4, 5) P2 to PI (3, 4, 5) P3 on the inner leaflet of the plasma membrane. They are further classified to class IA and class IB (Fry, 1994; Vanhaesebroeck and Waterfield, 1999). Class IA PI3K as heterodimeric molecules consist of a P110α, β, or δ (Cα, Cβ, Cδ) catalytic subunit (Domin, 1997) and one of the five relevant p85α (R1), p55α (R1), p50α (R1), p85β (R2) or p55γ (R3) regulatory subunits (Okkenhaug and Vanhaesebroeck, 2001). P110α and P110β are widely expressed in different tissues but P110δ is primarily expressed in leukocytes(Zhao, 2008; Domin, 1997).

Although class II enzymes are structurally related to class I, unlike classes I and III, comprise no more relevant regulatory proteins. They are so called PI3K C2 as they contain a C-terminal region with

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homology to C2 domains. PI3K C2 catalyses the production of PI (3) P and PI-3, 4-P2 in vitro (Arcaro et al., 1998; Hawkins et al., 2006). Three human subclasses for class II enzymes have been reported; PI3KC2α, PI3KC2β and PI3KC2γ. While C2α and C2β are expressed in all mammalian tissues(Maria-Magdalena Georgescu, 2011; Arcaro et al., 2000; Domin et al., 1997), C2γ is mainly expressed in prostate, liver and breast(Ho et al., 1997; Rozycka et al., 1998)

Class III PI3Ks enzymes generate PI3P from PI (Volinia et al., 1995). They consist of a catalytic Vps34 (C3) subunit and a regulatory p150 (R4) subunit and seem to be primarily involved in intracellular trafficking process of proteins and vesicles (Maria-Magdalena Georgescu, 2011; Foster et al., 2003; Schu et al., 1993; Vanhaesebroeck et al., 2001).

Prostate cancer is the most common cancer diagnosed in males all over the world. It accounts for the second principal cause of cancer deaths in men (Fritz H. Schröder, 2012; Hughes et al., 2005). Metastatic prostate cancer will spread beyond the prostate gland through the body. While the primary affected organ can effectively be cured by surgery or radiation therapy, therapeutic strategies are limited for metastatic disease (Silvia Letašiová, 2012; Ross et al., 2002). Bladder cancer is the second most common cancer of the genitourinary tract, approximately 30% to 70% of superficial bladder tumors will recur after initial treatment and 10% to 30% will progress to invasive and/or metastatic disease(Lee et al., 2002). Consequently the investigation of the molecular mechanisms involved in the emergence of metastatic genitourinary cancers is of great importance.

PI3K has been shown to be involved in the secretion of matrix metalloproteinases which degrade extra cellular matrix and major components of basement membrane and which are involved in cell invasion and cell migration(Brader and Eccles, 2004). Considering the pivotal role of PI3K signaling in cancer metastasis, broad-range PI3K inhibitors have been developed. Although they have shown early signs of becoming effective anticancer drugs, isoform specific inhibitors would provide further therapeutic advantage by limiting undesirable effects of pan-inhibitor compounds (Wee et al., 2008). In this regard identifying the most relevant isoforms in cancer tissues with activated PI3K pathway will be a prerequisite. Since it is unclear which PI3K isoform(s) drive growth and survival or invasiveness feature in human genitourinary cancers containing mutations in the PTEN tumor suppressor, PI3K negative

regulator; identification of the responsible downstream PI3K isoform will be important to overcome the challenge of restoring PTEN loss of function mutations in human cancers.

Materials and Methods

Cell lines and cell culture

The prostate carcinoma cell lines DU145 and PC3 and bladder carcinoma cell line 5637 (American Type Culture Collection, National Institute of Genetic Engineering and Biotechnology, Tehran, Iran) were grown in RPMI 1640 (Gibco, Brussel, Belgium) supplemented with streptomycin (Jabberebn Hayyan, Tehran, Iran), penicillin (Sigma, Brussel, Belgium), and 10% heat-inactivated fetal bovine serum (Sigma, Brussel, Belgium) in a humidified incubator under 5% CO_2 at $37°C$.

RNA isolation

Cells in subconfluent cultures were lysed in Tripure RNA-isolating reagent (Roche, Germany). All samples were extracted according to manufacturer instruction. Resulting purified intact RNA was treated with DNase I (Promega, USA) to remove genomic DNA contaminants. The RNA samples were then stored at -80°C. Experiments were performed at least three times using separate sets of cultures.

Primer for PI3K isoforms and housekeeping genes

Using criteria required for designing SYBER green assay, primers were designed using beacon designer software 6.0. To ensure the absence of secondary structure, mfold zuker program was run for the designed primers (Markham, 1995-2013). Additional criteria for a good SYBR green Real-Time assay included a relatively short amplicon (< 200 bp) for housekeeping genes and genes of interest. A blast search was performed to check the specificity of DNA sequences of primers. To amplify18SrRNA, the offered primers by Bio-rad were used (Table 1).

Table 1 : Sequences of primers and amplicon's length

Isoform	Primer	Size of product
PIK3CA (Cα)	Forward: AAAGTGTGTGGATGTGATGAATAC Reverse: GCTGTGGAAATGCGTCTGG	185 bp
PIK3CB (Cβ)	Forward: GACATCTGGGCGGTGGATTC Reverse: AAGGAGGTTGAACATTGGGTAATTG	171 bp
PIK3CD (Cδ)	Forward: TTCAGACACCATCGCCAACATC Reverse: ACAATAGCCAGCACAGGAGAGG	160 bp
PIK3CG (Cγ)	Forward: CCCGAAAGCCAAAGCGAACAG Reverse: GCCACTCTTCCTTCCTCACCTC	187 bp
PIK3C2A (C2α)	Forward: AAGTCAGCAATCTACAGGTATCTCC Reverse: TCAAGAAGAACAGCATCCCAAGG	175 bp
PIK3C2B (C2β)	Forward: CTGCGGGCTGGAGGAGTTC Reverse: GGAGATGGACGAGGTAGTTCAAGG	188 bp
PIK3C2G (C2γ)	Forward: AAGTGGCAGTTCAACAATTAGAC Reverse: CAGTAAAGACGATGGGCAACC	166 bp
PIK3R1	Forward: TGTCTGAACGGCTGAATATGAATAG Reverse: GTGTAATGTGAGGTCCCAATGC	175 bp
PIK3R2	Forward: AAGGGAGGAGGTGAACGAGAAAC Reverse: AACGGAGCAGAAGGTGAGTGG	187 bp
PIK3R3	Forward: ACAGATGCCCTCGCTTTGC Reverse: GCCTCTCCACTTCACATTCAC	160 bp
PIK3R5	Forward: GGAGGAGAGCACCAATGACATC Reverse: CAAAGCGGCAGTAGTAGAGTAGC	197 bp
PIK3C3	Forward: CCTTCAGTCTCTTCACCTCCTC Reverse: TCTTCACATTCCACTATCACATACC	158 bp
GUSB	Forward: CACGACACCCACCACCTACATC Reverse: GACGCACTTCCAACTTGAACAG	121 bp
18S rRNA	Forward: CGGCGACGACCCATTCGAAC Reverse: GAATCGAACCCTGATTCCCCGTC	99 bp
GAPDH	Forward: GCAGGGGGGAGCCAAAAGGGT Reverse: TGGGTGGCAGTGATGGCATGG	219 bp

Quantitative Real-Time RT-PCR

Real-Time PCR was performed in 25 µL of a reaction consisting of 21 µL Superscript III Platinum_R SYBR Green one-step RT supermix (Invitrogen, Belgium), 1 µM concentration of each primer and 2 µL CDNA as template. The PCR thermal profile consists of an initial incubation of 30 min at $50°C$ and 10 min at $95°C$ followed by 30 cycles of $95°C$ for 30 s, $54°C$ for 30 s, $72°C$ for 30 s, and $80°C$ for 10 s (data acquisition point). Amplification, detection, and data analysis were performed with MYiQ real-time detection system (Bio-rad, Milan, Italy). Each sample was run at least in duplicate. Relative quantitation of gene expression has been estimated according to the $2^{-\Delta\Delta Ct}$ method based on the threshold cycle (Ct) values(Schmittgen and Livak, 2008).

Statistical analysis

The Real-Time RT-PCR analysis was performed at least in 3 independent experiments. Each sample was run and examined in duplicate. Differences between groups were analyzed using the t-test. $P < 0.05$ was considered statistically significant.

Table 2: Location of the genes and primers

Gene type	Chromosome	Band	Primer Located Exon
PIK3C2A	11	p15.5—14	1
PIK3C2B	1	q32	6,8
PIK3C2G	12	p12	5,7
PIK3C3	18	q12.3	12,14
PIK3CA	3	q26.3	3
PIK3CB	3	q22.3	1,2
PIK3CD	1	p36.2	20,21
PIK3CG	7	q22.3	2
PIK3R1	5	q13.1	V1: 15 V2: 10
PIK3R2	19	q13.2-q13.4	7,9
PIK3R3	1	p34.1	10
PIK3R5	17	p13.1	12,13

Results

PI3K isoforms Gene Expression

Based on data obtained from Real-Time PCR, while Cα, Cβ, C2α, R1, R2 and R3 isoforms were expressed by PC3, DU145 and 5637 cell lines Cδ, Cγ , C2β, R5, C2γ and C3 isoforms were not detected in aforementioned cell lines(Table 3).

Table 3: Type of P13K isoforms demonstrated in DU145, 5637and PC3 cell lines

PK13 isoform/ cell lines	Cα	Cβ	C2α	R1	R2	R3	Cδ	Cγ	C2β	R5	C2γ	C3
DU145	+	+	+	+	+	+	-	-	-	-	-	-
PC3	+	+	+	+	+	+	-	-	-	-	-	-
5637	+	+	+	+	+	+	-	-	-	-	-	-

Ratio of the expression of PI3K isoforms in DU145 with respect to PC3

The expression level of Cα , Cβ, C2α, R1, R2 and R3 isoforms in DU145 cells were normalized against PC3 cells(null for PTEN gene). It showed that while in DU145 cells, R3 transcripts was decreased to about 80% of its amount in PC3 cells, Cα and R2 transcripts represented an equal expression for the compared cell lines. When the level of Cβ, C2α, R1 transcripts in DU145 cells were assessed, a significant increase in their relative expression was determined (Figure 1).

The expression level of PI3K isoforms in 5637 cells with respect to PC3

Looking to the result of the analysis of P13K isoforms, the data revealed increased quantity of Cα, Cβ, R2, R1 and C2α transcripts in 5637 cells. The highest increased value was detected for the R2 transcripts, while a little decrease in the level of mRNA transcripts was detected for R3 isoform (Figure 2).

Figure 2. Bar graphs showing the relative gene expression of PI3K isoforms in 5637 cell line. Total RNA was isolated and the relative gene expression of genes of interests were analyzed by Real-Time PCR. Relative quantitation of gene expression was estimated according to the 2-ΔΔCtmethod based on the threshold cycle (Ct) values. Each sample was assessed in at least duplicates.

Relative expression level of PI3K isoforms against Cα transcripts

When we compared 5637 and DU145 cell lines against PC-3, we detected the minimum change in the amount of PI3K-Cα transcript amongst the catalytic isoforms (Figures 1 and 2). So we calculated the ratios of interested transcripts of PI3K isoforms respected to the PI3K-Cα amount in the same cell line to search for any prominent inequality in expression for regulatory subunits respected to catalytic isoforms. Data for DU145 cell line showed a significant increase in PI3K-Cβ and PI3K-C2α catalytic isoforms with respect to all the three detected regulatory subunits (R1, R2, R3) (Figure 3).
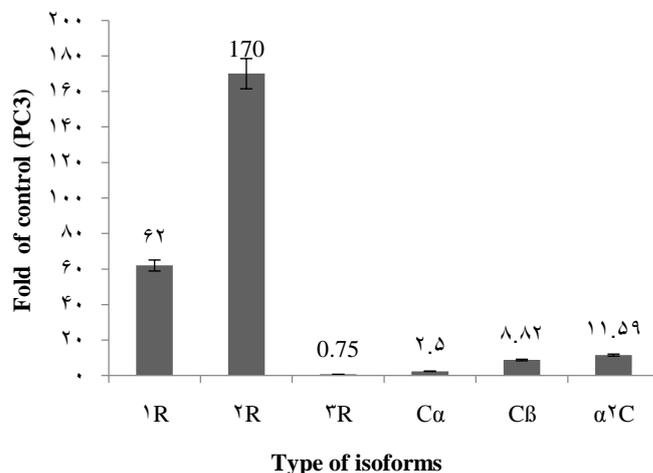
Figure1. Bar graphs showing the relative gene expression of PI3K isoforms in DU145 cell line. Total RNA was isolated and the relative gene expression of genes of interests were analyzed by Real-Time PCR. Relative quantitation of gene expression was estimated according to the 2-ΔΔCtmethod based on the threshold cycle (Ct) values. Each sample was assessed in at least duplicates,

Figure 3. Bar graphs showing the relative gene expression of PI3K isoforms which were normalized against Cα transcript in Du145 cell line. Total RNA was isolated and the relative gene expression were analyzed by Real-Time PCR. Relative quantitation of gene expression was estimated according to the $2^{-\Delta\Delta Ct}$ method based on the threshold cycle (Ct) values. Each sample was assessed in at least duplicates.
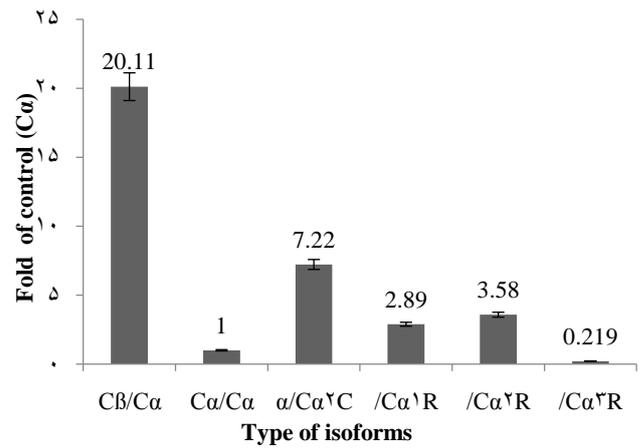
Figure 5. Bar graphs showing the relative gene expression of PI3K isoforms which were normalized against Cα transcript in PC3 cell line. Total RNA was isolated and the relative gene expression were analyzed by Real-Time PCR. Relative quantitation of gene expression was estimated according to the $2^{-\Delta\Delta Ct}$ method based on the threshold cycle (Ct) values. Each sample was assessed in at least duplicates.

For 5637 cell line data showed a significant increase in R2 and a reasonable increment in PI3K-C2α, PI3K-Cβ and R1 respected to PI3K-Cα catalytic Isoform. R3 revealed neglectable increment in expression (Figures 4).

In PC3, PI3K-Cβ and PI3K-C2α relative expressions were the same as detected for DU145 and 5637. However the regulatory subunits were increased as for 5637 cell line (Figure 5).
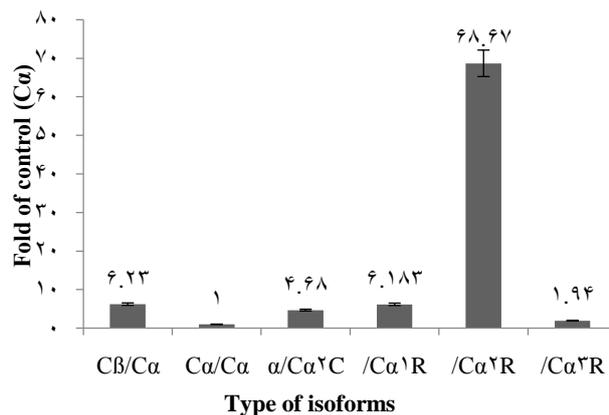
Figure 4. Bar graphs showing the relative gene expression of PI3K isoforms which were normalized against Cα transcript in 5637 cell line. Total RNA was isolated and the relative gene expression of genes of interests were analyzed by Real-Time PCR. Relative quantitation of gene expression was estimated according to the $2^{-\Delta\Delta Ct}$ method based on the threshold cycle (Ct) values. Each sample was assessed in at least duplicates

Discussion

Phosphatidylinositol 3-kinase (PI3K) pathway has a central role in cancer cells proliferation, survival and migration (Osaki et al., 2004). Considering the studies indicating a component of PTEN/PI3K/Akt cassette is altered in a large number of tumors it is believed that PI3K makes a great contribution to a high percentage of cancers (Fry, 2001). Consequently PI3K family as drug targets has been under investigation (Stein and Waterfield, 2000). PTEN encodes a phosphatase against both lipid and protein substrates. The mechanism by which PTEN might act as a tumor suppressor gene may involve inhibition of the PI3K-Akt signaling pathway which is essential for cell cycle progression and survival.

PTEN is expressed in prostate normal epithelial cells. Its expression mostly reduced in prostate cancer and the loss of expression occurred in cancers with high grade or stage (Fry, 2001).

The expression of PTEN is also negatively correlated with bladder tumor grades; reduced expression of PETN might play an important role in carcinogenesis and progression of bladder cancer (Zhang, 2005).

DU-145 (prostate cell line) and 5637 (bladder cell line) have functional PTEN tumor suppressor gene, unlike this feature these PTEN bearing cell lines display highly metastatic behavior like PC-3(prostate cell line), which is null for PTEN (Bastola et al., 2002; Wang et al., 2000). In pursuit

to explain why despite of bearing functional PTEN, DU145 and 5637 are highly invasive cell lines we searched for any discrepancy in PI3K isoforms expression rate and expression pattern against DU145, PC-3 and 5637 cell lines.

We investigated the expression profile of the twelve known isoforms from PI3K family including PI3K-Cα, PI3K-Cβ, PI3K-Cγ, PI3K-Cδ, PI3K-C2α, PI3K-C2β, PI3K-C2γ, C3, R1, R2, R3, R5 isoforms. To perform precise quantitative Real-Time PCR analysis, GAPDH, 18srRNA and GUSB were included in experiments as control housekeeping genes (HKGs). Our results showed from the three studied HKGs, 18srRNA and GAPDH were the most reliable genes as reference controls and the GUSB gene revealed unstable expression in studied cell lines.

Whereas PI3K-Cα, PI3K-Cβ, PI3K-C2α, R1, R2 and R3 were the most frequently expressed isoforms in all the three studied cell lines; PI3K-Cγ, PI3K-Cδ, PI3K-C2β, PI3K-C2γ, C3 and R5 isoforms were found to be undetectable.

When PC-3 cell line was used as calibrator evaluation of quantitative gene expression demonstrated that PI3K-Cα and R2 represented an equal amount of transcripts for both DU145 and PC-3 cell lines, the expression ratio for R3 showed 80% decrease in DU145 and the ratio of gene expression for R1, PI3K-Cβ and PI3K-C2α were increased to 3.73, 7.69 and 3.12 fold respectively.

When we compared 5637 cell line against PC-3(as calibrator) the ratio of gene expression were 62.16, 170, 0.72, 2.5, 8.83 and 11.59 fold for R1, R2, R3, PI3K-Cα, PI3K-Cβ and PI3K-C2α respectively.

It is speculated that more consequences of PI3Ks in disease will be well-defined by array based PI3K gene profiling and subsequently promised that pharmaceutical intervention will develop isoform-specific PI3K inhibitors with clinical benefits to human (Vanhaesebroeck and Waterfield, 1999).

It has been shown that in cervical and ovarian cancers gene amplification of PI3K-Cα isoform culminated to its over-expression. On the other hand in ovarian and colon tumors oncogenic activating mutations were detected in R1 gene (Sawyer et al., 2003). However there exists little information about typical distribution and quantitative expression of all the family members of PI3K isoforms in normal and diseased states.

Among members of PI3K family, class I have been studied extensively. Results of gene expression analysis have suggested that both PI3K-Cα and PI3K-Cβ are widely expressed in mammalian tissues. Further experimental evidence documented the equimolar expression of catalytic and regulatory subunits in studied tissues (Geering et al., 2007). Further compelling evidence revealed that R2 protein reactivity was higher than R1 isoform when compared for WEHI-231 and NIH 3T3 cell lines (Geering et al., 2007). This finding is completely coinciding with our data which has demonstrated high level of R2/R1 expression ratio for DU145, PC3 and 5637 cells. Other recent researches showed an increased specific activity for PI3K-Cα when compared with the other members of class IA. This could explain why its expression ratio is lower than the other catalytic subunits in our studies.

Conclusion

R1, R2, PI3K-Cα, PI3K-Cβ and PI3K-C2β isoforms in 5637 cell line and R1, PI3K-Cβ and PI3K-C2α isoforms in DU145 were increased in these both PTEN bearing cell lines respected to PC3 which is null for PTEN. To shed more light on the functional significance of isoforms over-expression we propose to silence the aforementioned isoforms by RNA interference. It should also be considered that rather than PI3K malignant activation, the extra-activation of other pathways like MAPK and PKC may bypass PTEN normal function for PI3K regulation in 5637 and DU145.

Acknowledgment

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Open Access Statement:

Cloning and Expression of Fusion (F) and Haemagglutinin-neuraminidase (HN) Epitopes in Hairy Roots of Tobacco (*Nicotiana tabaccum*) as a Step Toward Developing a Candidate Recombinant Vaccine Against Newcastle Disease

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Abstract

Newcastle is a significant avian disease continuing to cause considerable loss. Developments in genetic engineering have led to plant-based platforms for human and animal vaccine production. Recombinant vaccine production in hairy root systems have several advantages over stable expression in whole plants, including high growth rates, ready genetic manipulations, high levels of recombinant protein production, and the potential for bioreactor culture. In an attempt to develop a recombinant vaccine in hairy roots, the sequences encoding fusion (F) and haemagglutinin-neuraminidase (HN) epitopes of Newcastle disease virus were cloned in pBI121 expression vector which was then transferred into leaf disks of tobacco (*Nicotiana tabaccum*) 'Turkish' cultivar by means of *Agrobacterium rhizogenes*. Hairy roots developed on MS medium containing 50 mg/L kanamycin and 30 mg/L meropenem. Incorporation of the heterologous gene in the genome of hairy roots was confirmed by PCR. Expression analyses were performed by real-time PCR at transcription level and by dot-blot and ELISA assays at translation level, all confirming the expression of the heterologous gene and production of the recombinant protein.

Keywords: Recombinant Vaccine, Antigen Expression, Newcastle Disease, Hairy Roots, *Agrobacterium Rhizogenes*

Introduction

Recombinant vaccines based on viral coat protein subunits represent an efficient tool as a substitute for conventional, attenuated virus based vaccines (Makela, 2000). They are called edible recombinant vaccines as they are administered orally. A recombinant (or subunit) vaccine is an antigen produced with the help of genetic engineering methods in a suitable expression system. Theoretically, the genes encoding every protein can be cloned and expressed in bacteria, yeasts or mammalian cells. A number of genes encoding surface antigens from viruses, bacteria and single-celled pathogens have been cloned in expression systems and the expressed antigens have been used as vaccines (Arntzen and Mason, 1995). Plant-based platforms are one of the highly preferred expression systems employed for recombinant vaccine production (Sala et al., 2003). Transgenic plants expressing industrially or pharmaceutically valuable heterologous proteins can well be a substitute for fermented systems. Several vaccines have been produced on a transient or stable basis in plants and proved to conserve the necessary conformation for evoking the immune response in human or animals.

Transgenic plants are highly interesting candidates for recombinant vaccine production. The main benefits of plant-based edible vaccines include ease of production and administration, as well as a very high level of immunogenicity because they elicit the mucosal immune system, which is the first and most important barrier against different pathogens. The majority of animal pathogens enter the host's body through respiratory or digestive tracts, where the mucosal immune system operates; and the mucosal immune response elicited by edible vaccines will have a very high potency against pathogens (Arntzen and Mason, 1995). Production of recombinant vaccines against veterinary diseases such as Norwalk virus and foot and mouth disease has been reported by several researchers (Mason et

al., 1996; Lai et al., 2012). In each case, epitopes evoking the immune system have been used in bacteria, yeast or plant-based expression platforms (Mason et al., 1996; Lai et al., 2012), among which the plant-based systems possess a number of advantages as follows: 1- the potential to elicit humoral, cell-mediated, and mucosal immune systems; 2- plant cell walls preserve the antigen from digestion, and it will be released gradually into lymph and later into blood; 3- packaging of viral antigen in plants is exactly similar to that observed in diseased individuals, assembling into subviral particles (Sala et al., 2003); 4- the costs of vaccine production in transgenic plants are considerably lower than conventional methods, with the costs being limited to those of typical plant cultivation and harvest; 5- plant parts or extracts can be kept at room temperature without any special equipments; 6- plants are not hosts of animal viruses, therefore eliminating the risk of mammalian virus contamination; 7- vaccines produced in plants can be consumed as a dietary supplement (Kim and Yang, 2010).

There are two methods for incorporating antigens into plant genomes and producing recombinant vaccines (Fieler et al., 1997). The first method includes insertion of the antigen coding gene into nuclear or organellar genome, resulting in a stable transgenic plant. In the second method, the gene is transferred to the plant by means of *Agrobacterium* or a plant virus, and eliminating the need for *in vitro* tissue culture and plant regeneration (Waugh, 2005).

Expression of recombinant vaccines in hairy roots mediated by *A. rhizogenes* has several advantages over stable expression in whole plants. Among these advantages are high growth rates, low duplication times, easy genetic manipulation, high capacity for recombinant protein production, the potential for growth in bioreactors, the possibility of evaluating expression courses and levels, and verification of the constructs without the need for development of stable transgenic plants (Giri and Narasu, 2000; Fischer and Schilberg, 2004).

Hairy root production in tobacco is preferred over other plants due to several factors such as ease of transformation, availability of optimized tissue culture protocols, and relatively high levels of transgene expression (Tripurani et al., 2003; Arntzen and Mason, 1995).

Newcastle virus is a prominent avian pathogen, both epidemiologically and economically. Killed or attenuated virus is currently used for Newcastle vaccination (Zhao and Hammond, 2005), often forming an effective solution. High costs and the risk of improper attenuation however necessitate a new means of vaccination against Newcastle disease (Zhao and Hammond, 2005).

Newcastle disease virus (NDV), a member of the *Rubulavirus* genus in the *Paramyxoviridae* family, is an enveloped virus with a single-stranded, monopartite anti-sense RNA genome of 15 kb length. Its genome encodes 6 principal structural and non-structural proteins including nucleocapsid (NP), phosphoprotein (P), matrix (M), fusion (F), haemagglutinin-neuraminidase (HN) and RNA-dependent RNA polymerase (L) (Berinstein et al., 2005).

F and HN are trans-membrane glycoproteins necessary for infectivity and pathogenicity and both of them can evoke immune response (Berinstein et al., 2005). Amino acid residues 65 to 81 of F and 346 to 353 of HN are known to constitute the most influential immunogenic sites for antibody induction (Zhao and Hammond, 2005).

The objective of this work was to transform and express the F and HN epitopes in tobacco hairy roots. Tobacco is a model organism in transgene expression studies, and successful expression of antigens in its hairy roots could be a step toward recombinant vaccine production in other plants used as avian feed.

Materials and Methods

Design and preparation of the construct

The construct used in this work included 4 tandem repeats of the HN epitope (encoding residues 346-353 of the HN protein) followed by 3 tandem repeats of the F epitope (encoding residues 65-81 of the F protein). The sequences were retrieved from the NCBI database, and optimized according to the codon bias of the host plant in order to guarantee a high level of expression in its hairy roots.

After optimization, the sequence was examined with the Lasergene software (DNASTAR) to ensure the absence of any transcription or translation inhibitor sequences. A histidine tag (18 bp) was then ligated upstream the construct, followed by the omega sequence (67 bp) as the ribosome attachment site. A 18 bp sequence encoding the endoplasmic reticulum retention signal peptide (SEKDEL) was also fused to the 3' terminus. The omega sequence (Berinstein et al., 2005) and signal peptide (Kang et al., 2004) serve an important role in enhancing the expression level of epitopes.
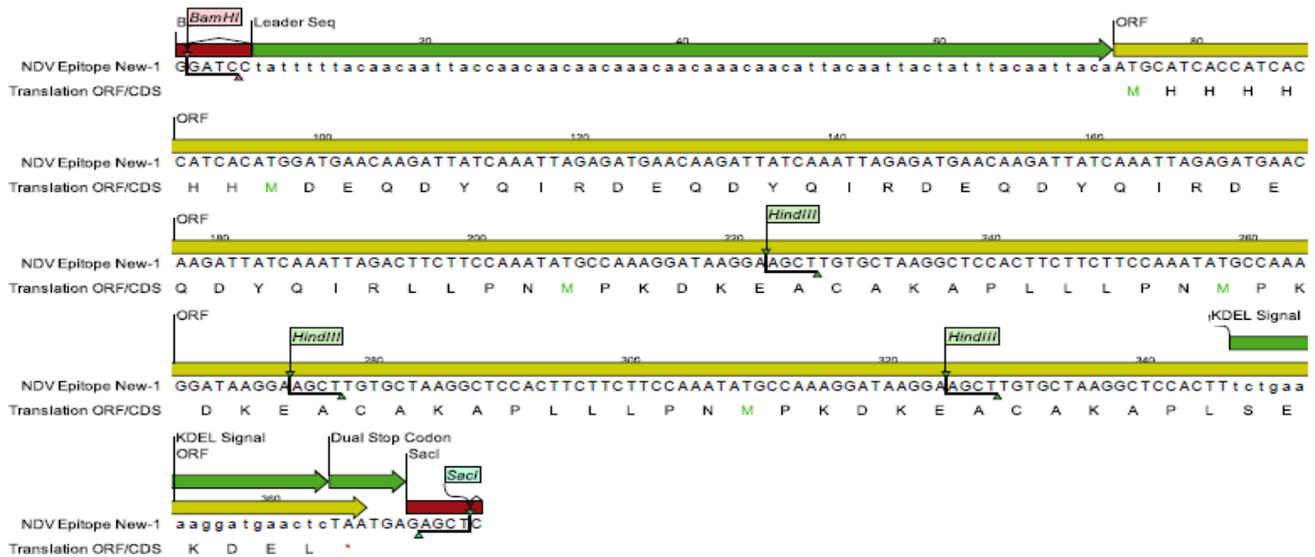
Figure 1. Expression cassette incorporating the F and HN epitopes together with the other sequences necessary for expression in eukaryotic systems

Finally, two restriction sites, *Bam*HI and *Sac*I, were incorporated at 5' and 3' termini respectively to facilitate the cloning process. These elements increased the size of the cassette to 376 bp. The resulting F-HN cassette (fig. 1) was designed with CLC software package, synthesized by Gene Ray, and cloned into pGH vector.

Preparation of pBI121 vector carrying F and HN epitopes for transformation of the nuclear genome

The F-HN cassette was extracted from pGH plasmid by enzymatic digestion with *Bam*HI and *Sac*I. pBI121 binary vector harbouring CaMV 35S promoter and NOS terminator was also digested at the same sites. After elimination of the GUS sequence, the F-HN cassette was inserted between promoter and terminator (fig. 2).
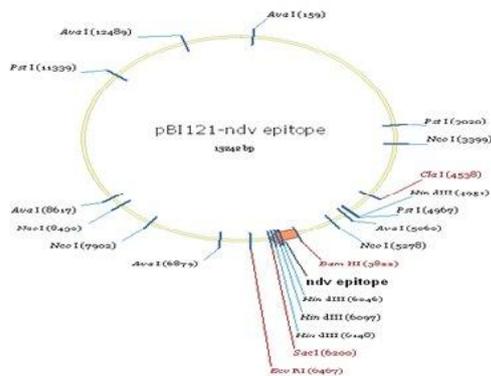
Figure 2. Schematic structure of the recombinant plasmid pBI121- NDV epitope for Agrobacterium-mediated transformation of tobacco hairy root.The DNA sequence encoding NDV epitope was cloned the downstream of the CaMV 35S promoter of a binary plasmid pBI 121

The resulting construct (named pBI121-NDV epitope) was introduced into *A. rhizogenes* by electroporation, and its proper placement downstream the promoter was confirmed by plasmid extraction and digestion with *Bam*HI and *Sac*I, which gave a 376 bp band on the electrophoresis gel (fig. 3).
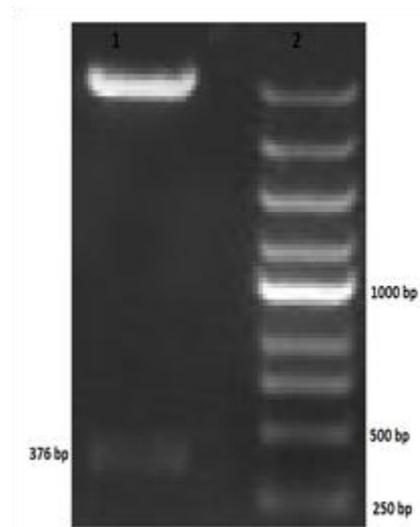
Figure 3. pBI121-NDV epitope extracted from *A. rhizogenes*, digested with *Bam*HI and *Sac*I: digested plasmid (left) and size marker (right)

Generation of transformed hairy roots

A. rhizogenes strain Ar15834 carrying the construct (pBI121-NDV epitope) was cultured in Luria-Bertani (LB) medium for 18 hours, to an optical density (OD) at 600 nm of approximately 0.6%. Suspensions were centrifuged at 3500 rpm for 20 minutes at 4°C. The pellets were suspended in MS medium (pH 5.2) with 0.05 mMacetosyringone (3′, 5′-dimethoxy-4′-hydr -

oxyacetophenone) and injected into tobacco leaves with insulin syringe.

Transformed tobacco leaves were cultured on MS medium (Murashige and Skoog, 1962) with no plant growth regulators (co-culture medium), and subcultured on MS medium containing 50 mg/l kanamycin and 30 mg/l meropenem (selection medium) upon the emergence of bacterial colonies around them. Hairy roots were generated in ample quantities within 35 days (fig. 4), and subcultured in liquid, hormone-free MS medium.

Figure 4. Generation of transformed hairy roots on MS medium containing 50 mg/l kanamycin and 30 mg/l meropenem

Molecular verification of hairy roots

Molecular verification of hairy roots was achieved by PCR amplification of a 194 bp fragment of the rolB gene. Genomic DNA was extracted from hairy roots following Dellaporta et al. (1983), and PCR reaction was performed with the following primers:

Forward:
5'AAGTGCTGAAGGAACAATC 3'
Reverse:
5' CAAGTGAATGAACAAGGAAC 3'

PCR cycles comprised denaturation at 94°C for 1 minute, annealing at 51.5°C for 30 seconds, and extension at 72°C for 20 seconds.

Examination of transformed hairy roots for the presence of F and HN epitopes

Transformation of hairy roots was confirmed by PCR. Genomic DNA was extracted following Dellaporta et al. (1983). A 741 bp fragment was amplified with the following specific primers:

Forward:
5' ATGAACAAGATTATCAAATTAGAC 3'
Reverse:
5' CCGTAAAGCACTAAATCG 3'

The 35 PCR cycles comprised denaturation at 94°C for 1 minute, annealing at 46°C for 1 minute and extension at 72°C for 1 minute. Genomic DNA from untransformed hairy roots was used as the negative control.

Expression analysis at transcription level with real-time PCR

Quantitative Real-Time PCR experiments were performed in duplicate for each sample to quantify the expression level of the transgene. Total RNA was extracted from the transformed hairy roots. cDNA was constructed by reverse transcription using oligo-dT primers and used as the real-time PCR template. Real-time PCR reaction volumes of 20 μl included 0.5 μl of each primer and 10 μl SYBR Green Real time PCR master mix., and the following specific primers:

Forward:
5' GACTTCTTCCAAATATGCCAAAG 3'
Reverse:
5' TGGAGCCTTAGCACAAGC 3'

Expression analysis at translation level with dot-blotting

Production of the recombinant protein in tobacco hairy roots was verified by dot-blotting. A protein sample of 3 μl volume was spotted onto the membrane and allowed to dry at 37°C. The membrane was incubated for 1 hour with blocking solution (BSA) which prevents non-specific reactions, then incubated for 1 hour with anti-His tag, rinsed 3 times with PBST/PBS, incubated for 1 hour with the secondary antibody (at 37°C), rinsed 3 times with PBST/PBS, and finally incubated with OPD substrate. A 3 μl sample of commercial NDV vaccine and the same volume of the protein from untransformed plants were used as positive and negative controls, respectively.

Quantification of protein expression with ELISA assay

Expression of the heterologous protein was evaluated by ELISA assay. ELISA plate was coated with total soluble proteins from transformed and untransformed hairy roots and the NDV antigen at 37°C for one hour and then incubated with 1% BSA in PBS at 37°C for 2 hours in order to prevent non-specific binding. The wells were washed by PBST/PBS, incubated with anti-His tag (1:1000 dilutions) and then with alkaline phosphatase conjugated with anti rabbit IgG (1:1500 dilution), and finally developed with TMB substrate. Colour reaction was stopped by adding 2N H_2SO_4 and read at a wavelength of 405 nm.

Results

In this research transformed hairy roots were obtained by co-culture of tobacco leaf disks with *A. rhizogenes* carrying the binary vector pBI121-NDV epitope. PCR amplification of a 194 bp fragment of the rolB gene confirmed that the generated hairy roots are a result of A. rhizogenes infection (fig. 5).

Figure 5. Molecular verification of hairy roots by amplification of a 194 bp fragment of the rolB gene. Lane 1 is 100bp size marker; lanes 2-8 are the amplified 194 bp rolB fragment

A second PCR confirmed the presence of the construct carrying F and HN epitopes in the transformed hairy roots (fig. 6). As shown in fig. 6, the 741 bp band is only observed in transformed hairy roots.
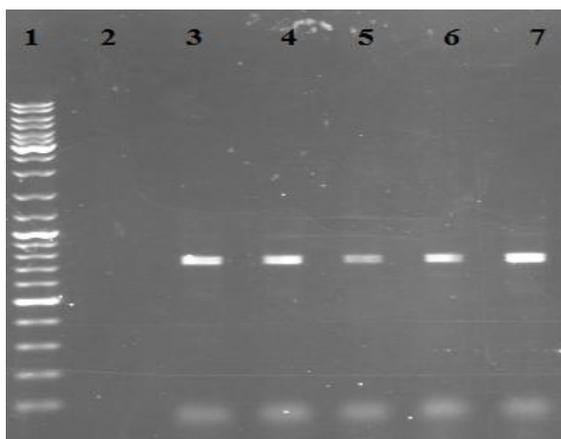
Figure 6. Verification of the presence of the construct harbouring F and HN epitopes in hairy roots by PCR amplification of a 741 bp fragment of the construct: 100bp size marker (1), untransformed hairy root as negative control (2), plasmid pBI121-NDV as positive control (3) and transformed hairy root (4-7)
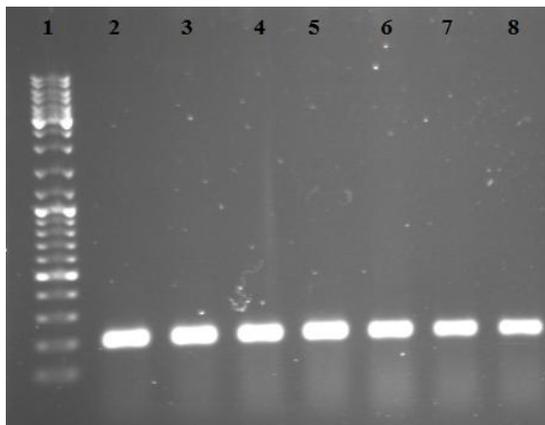
Expression of the construct harbouring F and HN epitopes was quantified at transcription level with real-time PCR. The results indicated that the

transgene is expressed in transformed hairy roots (chart 1).
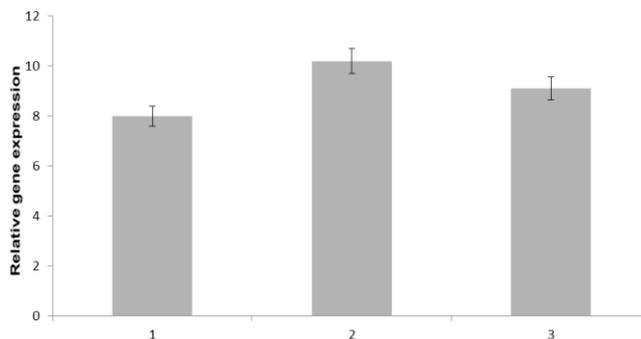
Chart 1. Expression analysis of the construct carrying F and HN epitopes at transcription level in transformed hairy roots by means of real-time PCR (1,2 and 3 are transformed hairy root samples)

Recombinant protein production was verified by dot-blot assay, confirming that the recombinant protein is produced in transformed, but not in untransformed hairy roots (fig. 7).

Figure 7. Verification of recombinant protein production in transformed hairy roots with dot-blotting: positive control (1); protein from transformed hairy roots (2); wild-type protein as negative control (3)

Recombinant protein production was also quantified by ELISA, with the results indicating production of very high levels of recombinant protein in transformed hairy roots, whereas untransformed roots were lacking a cogent evidence of recombinant protein production (chart 2).
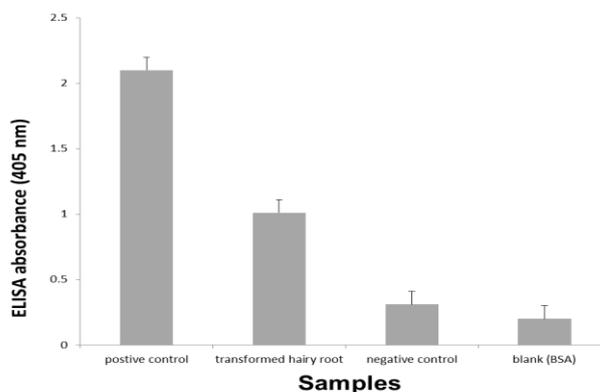
Chart 2. ELISA results showing production of F and HN epitopes in hairy roots. From left to right: commercial vaccine as positive control; transformed hairy root; untransformed hairy root as negative control; BSA as blank sample

15

Discussion

Green plants represent appropriate systems for expression of recombinant vaccines and other pharmaceutical proteins; but several factors such as the considerably long time required for development of transformed lines, relatively high costs, and low expression levels remain major obstacles to commercialize recombinant vaccine production using *A. tumefaciens*-mediated plant genetic engineering (Bendahmane et al., 2000).

expression of antigens in hairy roots or in whole plants using plant viral vectors has been proposed as a solution to these pitfalls. Although expression in hairy roots is not a suitable method for commercial production of recombinant vaccine in plants, the antigens produced in this procedure can be used for production of specific antibodies required in molecular diagnosis. Moreover, this method makes it possible to evaluate efficacy of a potential recombinant vaccine in a short time.

Expression in hairy roots involves introduction of the antigen coding sequence into the plant tissue by means of *A. rhizogenes*, and induction of transformed hairy root production.

In the present work, F and HN epitopes of Newcastle disease virus were expressed in tobacco hairy roots with the help of *A. rhizogenes*. As confirmed by Real Time PCR assay, expression level of the transgene was fairly high which is in agreement with the results obtained other investigators who had reported that foreign gene expression in transient gene expression assays is much higher than that of stable transformation (Leckie and Stewart, 2011; Wroblewski et al., 2005).

As indicated by dot-blot assay, the protein sample from transformed hairy roots produced an intense signal comparable to that from commercial Newcastle vaccines whereas protein of wild type plant was not detectable.

ELISA assay, which quantitates protein levels, also showed that the recombinant protein is produced at very high levels in transformed hairy roots. The faint absorbance observed for untransformed hairy root sample is probably resulting from the non-specific binding of proteins with the anti-histidine tag.

Plant viruses are also used as vectors for transient antigen expression in plants, where the desired epitope is inserted inside the gene encoding viral coat protein (Sala et al, 2003). Despite being a highly efficient method for recombinant protein production, preparation of viral vectors is a very time consuming and labour intensive task. Another drawback of viral vectors is that insertion of genes larger than a threshold size will diminish the efficiency of the vector (Sala et al., 2003).

On the other hand, the problems associated with recombinant protein production in stably transformed plants such as post-transformation gene silencing, undesirable alterations due to bond formation with complex carbohydrates, the considerable time required for development of a stable transgenic line with appropriate protein production capacity, and most importantly the environmental issues and the possibility of transgene escape (Jianxiang et al., 2007; Julian et al., 2003) have stimulated the application of alternative means such as transformed hairy roots for production of recombinant proteins or other products (Giding et al., 2000).

In the present work, different elements including the CaMV35s promoter, omega and SEKDEL sequences, and codon optimization were used in order to enhance the expression of F and HN epitopes, building on the published literature (Streatfield et al., 2001; Kang et al., 2004; Sala et al., 2003; Streatfield, 2005) reporting them to be important factors which increase expression levels of epitopes in the process of recombinant vaccine production.

It should be noted that the observed increase in expression levels can not be conclusively attributed to the mentioned elements, as the authors did not examine their influence; and this could be the subject of further studies.

Conclusion

Developments in genetic engineering have led to plant-based systems for recombinant vaccine production; but these systems are accompanied by a number of drawbacks which might be overcome with transient expression systems such as hairy roots. Here we report the application of tobacco hairy roots for expression of the F and HN epitopes of Newcastle disease virus. The results showed that hairy roots represent an efficient tool for expression of these viral antigens.

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Open Access Statement:

Association of miR-132 and miR-185 Genes Methylation and their Expression Profile with Risk of Congenital Factor XIII Deficiency

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Abstract

Congenital factor XIII deficiency is a very rare bleeding disorder, but because of the high rate of consanguineous marriages, it is common in Sistan and Baluchestan Province of Iran. The discovery of promoter hypermethylation of numerous miRNAs in human diseases has demonstrated an epigenetic mechanism for aberrant miRNA expression. The present study has analyzed methylation and expression status of miR-185 and miR-132 genes in patients with inherited factor XIII deficiency in a sample of South-Eastern Iranian population. Promoter methylation of miR-185 and miR-132 was investigated by Methylation Specific Polymerase Chain Reaction (MS PCR) in blood samples of 75 factor XIII deficient individuals and 74 healthy controls. Expression level of these genes was also assessed in 15 blood samples of patients and 15 healthy controls using real-time quantitative reverse transcription PCR. Analysis of miR-132 and miR-185 promoter hypermethylation did not show any significant difference between cases and controls. Relative gene expression analysis in cases (n=15) with congenital factor XIII deficiency and healthy controls (n=15) revealed no statistically significant relationship for miR-132 ($p = 0.126$) and miR-185 ($p = 0.165$) genes. Our findings indicated that promoter methylation as well as gene expression of miR-132 and miR-185 had no significant effect on etiology of factor XIII deficiency.

Introduction

Congenital factor XIII (FXIII) deficiency, which was first recognized by Duckert in 1960, is a rare bleeding disorder inherited as an autosomal recessive manner with an incidence of one per 2 million people in the general population. It is characterized by hemorrhagic diathesis frequently connected with spontaneous miscarriage and defective wound healing (Board et al., 1993; Muszbek et al., 2011; Ivaskevicius et al., 2007).

FXIII is expressed during compartmentalization of precursors of monocyte/macrophage and megakaryocyte/platelet cell lines in the bone marrow (Adany and Bardos, 2003). Plasma FXIII is a protransglutaminase, activated by thrombin and converted to transglutaminase in presence of calcium ions. It is a tetrameric molecule composed of 2 A-subunits and 2 B-subunits, which are noncovalently kept together in a heterologous tetramer. Subunit A with catalytic function is synthesized by hepatocytes, monocytes and megakaryocytes. Subunit B, which has no enzymatic activity and may serves as a plasma carrier molecule, is synthesized in the liver (Hsieh and Nugent, 2008; Ashcroft et al., 2000). In the cellular form and in tissues, the protein is only a homodimer of A subunits (A2). FXIII was first noted for its involvement in the coagulation cascade, where it covalently cross-links fibrin monomers and converts soft fibrin clots into stable hard clots. Factor XIIIa is able to cross-link α2-antiplasmin as well as extracellular matrix proteins such as vitronectin, fibronectin and collagen to fibrin, rendering the clot more resistant to lysis and anchoring the clot to the blood vessel wall, respectively (Takagi and Doolittle, 1974). MicroRNAs (MiRNAs) are considered as a regulator of platelet function. MiRNAs are small non-coding RNA molecules (22–25 nucleotides) regulating a range of biological processes by inducing RNA degradation and translation inhibition of targeted mRNAs by binding to complementary sites in the 3′-UTR of target transcripts via Watson-Crick base-pairing (Esquela-Kerscher and Slack, 2006). These molecules play pivotal roles in diverse gene regulatory pathways including timing control of developmental

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processes, hematopoietic cell differentiation, apoptosis, cell proliferation and organ development (Morita and Han, 2006; Robert et al., 2007; Amelia et al., 2005; Zhao, 2007). MiRNA deregulation can take place at both transcription and processing levels, and aberrant DNA methylation patterns have been implicated in altered expression of 30 or more miRNA genes in different diseases (Erik et al., 2011). Recently, they have also discovered that DNA methylation has emerged as an important mechanism in the regulation of MiRNA expression. Relatively little information is available on epigenetic regulation of miRNAs genes in coagulation disorders (Kunej et al., 2011). Recently, a role for miRNAs has been proposed in the regulation of innate immune responses in monocytes and macrophages. activation of the innate immune response has been associated with changes in the expression of some miRNAs including miR-132 (Taganov et al., 2006). Platelet-derived miRNAs could act as paracrine regulators of endothelial cell gene expression. activated platelets shed miRNAs including miR-185 that can regulate endothelial cell gene expression and a recent study demonstrated that platelet miRNA content of ST-elevation myocardial infarction (STEMI) patients is distinctly different to that of healthy individuals (Gidlöf et al., 2013).

In this study, we assessed methylation and expression profiles of miR-185 gene on chromosome 22 (22q11.21) and miR-132 gene on chromosome 17 (17p13.3) predicted to bind the 3' UTR of A-subunit (FXIII-A) protein in normal controls and patients with severe congenital factor XIII deficiency.

Materials and Methods

Diagnosis of and characteristics study population

In hemophilia center of Zahedan city all suspected to factor XIII deficiency were referred to coagulation laboratory of Iranian Blood Transfusion Organization (IBTO). In coagulation laboratory patients were assessed by clot solubility test in 5M urea or 1% monochloroacetic acid environments. Since three separated molecular studies on more than three hundred of patients in this center revealed that all of them were homozygote of FXIII-A Trp187Trp polymorphism, these patients were not again analyzed molecularly for this polymorphism (Naderi et al., 2014; Naderi et al., 2013). 75 patients as well as 74 age and sex matched healthy individuals were selected for assessment the promoter methylation status miR-

132 and miR-185 genes. Out of these two groups, two 15 persons groups were selected for the evaluation of miR-132 and miR-185 genes expression.

Bioinformatics

For the assessment the effect of miRNAs on factor XIII-A subunit we used algorithms of miRNA (http://www.microrna. org). According to these algorithms, we predicted that the FXIII-A gene might be a direct target of miR-132 and miR-185. Therefore the effects of these two miRNAs on factor XIII were assessed in this study.

Sample and DNA preparation

75 blood samples of patient with FXIII deficiency were collected from hemophilia center of Zahedan city, and 74 randomly control blood samples were obtained between November 2012 and May 2013 and all of them were age and sex matched. Informed consent was obtained from all the cases and controls and the study was approved by ethical committee of Sistan and Baluchestan University. Total DNA was extracted from frozen EDTA blood by using salting out method as described previously (Hashemi et al., 2014). Concentration of DNA samples was determined by spectrophotometer, and their integrity was assessed by gel electrophoresis. To modify DNA, 2 µg of extracted genomic DNA was incubated with 0.3 M NaOH for 20 min at 37°C and diluted with 550 µl of a 3.5 M sodium bisulfite (pH 5.0)and1 mM hydroquinone solution (both Sigma Aldrich), and then incubated at 55°C for 16 hr. The modified DNAs were then purified using a Wizard DNA Clean up System (Promega, Madison, WI). Purified DNAs were incubated with 0.3 M NaOH for 10 min at 37°C. DNA was precipitated with ethanol, and the treated DNA was diluted in 20 ml water, and was kept in −20 °C for use in later experiments.

Methylation Specific PCR (MSP)

Each PCR sample contained 1 µL bisulfite-modified DNA in a final volume of 25 ml including 16 µL RNase free double distilled water,0.5 µL Hot Star *Taq*® (Parstus: 2.5 U/ml), 1 µL dNTP mix (10 mmol/L), 2.5 µL 10× buffer, 1 µL each of primers (10 mmol/L), and 2 µL $MgCl_2$ (25 mmol/L). MSP amplification was set as follows: 94 °C for 10 min, followed by 39 cycles (94 °C for 30 s, the annealing temperature for miR-185: M=48/2, U=51; miR-132: M=51.8, U= 48.8 for 30 s and extension at 69 °C for 30 s). Final incubation was completed at 72 °C for 10 min. MSP products were separated by electrophoresis on 2% agarose gel and visualized

after ethidium bromide staining. The sequences of primers used for the methylation study are listed in Table 1.

Table 1. Primer sequences

miR-185 M	F: 5_TTTTTTTGTAGAGTTGGTTAATTCG_3
	R: 5_ATCACCATATCTTAATCTAAACGCA_3
miR-185 U	F: 5_TTTTTTTGTAGAGTTGGTTAATTTGG_3
	R: 5_ATCACCATATCTTAATCTAAACACA_3
miR-132 M	F: 5_TTTTTTGGGATATTTTTGACGTTAC_3
	R: 5_CCGACTAAAAACTCTACTACTCCG_3
miR-132 U	F: 5_TTTTTGGGATATTTTTGATGTTATG_3
	R: 5_CCAACTAAAAACTCTACTACTCCAC_3

M; methylated, U; unmethylated, F: Forward, R: Reverse

Gene expression analysis

Total RNA was extracted from fresh blood samples using bioZOL ™-B Blood RNA Isolation Kit (USA, bioWORLD.Cat No: 10760125-1) according to the manufacturer's instructions. MiRNA 1st-Strand cDNA Synthesis Kit (Agilent technologies, Cat. no. 600036) was used for elongating miRNAs in a polyadenylation reaction to reverse transcribe the polyadenylated RNA into QPCR-ready cDNA. 5SrRNA was used as an internal standard. ABI 5700 sequence detection system (Applied Biosystems) was used to estimate the quantity of cDNA using real-time quantitative PCR. The sequences of primers used for the expression study are listed in Table 2.

Table2. Real-time primer sequences

5Sr RNA (Real Time-PCR)	F: 5_CCAACGTCCATACCATGTTGA_3
	R: 5_GCGGTCACCCATCCAAGTA_3
miR-185 (Real Time-PCR)	F:5_GCGGCGGAGGGGGCGAGGGATTG_3
	R:5_ ATCCAGTGCAGGGTCCGAGG_3
miR-132 (Real Time-PCR)	F: 5_GCGGCGGCCGCCCCCGCGTCTC_3
	R: 5_ATCCAGTGCAGGGTCCGAGG_3

Statistical Analysis

Data were analyzed using SPSS software. The chi-square test was used for categorical variables, and the impact of methylation of miR-185 and miR-132 genes on the risk of congenital factor XIII deficiency was detected by estimating odds ratios (OR) and 95% confidence intervals (95% CI) using the binary logistic regression test. Analysis of relative gene expression ($2^{-\Delta\Delta CT}$) between patients and controls was done by independent t-test. The

significance level was set at p≤0.05 for all the tests.

Results
Characteristics of study population

All study population had an abnormal clot solubility test and molecular analysis of two separated studies on 190 and 70 patients revealed that all of them were homozygous of factor XIII Trp187Arg polymorphism. These 260 patients were comprised more than two third of patients with factor XIII deficiency in this center. The mean age of patients was 14/65±11/35 years while in control group was 15/19±10/45 years (*p* value: 0.76) (Table 3).

Table3. Demographic characteristics of 74 patients with factor XIII deficiency as well as 75 healthy individuals

Characteristic		Controls N = 74 (%)	Cases N = 75 (%)	*p*-value
Mean age		15/19 ± 10/45	14/65 ± 11/35	0/76
Sex	male	36 ± 0/50	34 ± 0/50	0/57
	female	38 ± 0/06	41 ± 0/06	
Family history	Yes	-	47 (62/67)	-
	No	-	28 (37/33)	
Intracranial bleeding	Yes	-	17 (22/67)	-
	No	-	58 (77/33)	
The first symptoms	Umbilical cord bleeding	-	31 (41/33)	-
	Another symptoms	-	44 (58/67)	
Age at diagnosis	0-5	-	50 (66/67)	-
	5-10	-	10 (13/33)	
	10≤	-	15 (20/0)	

Promoter methylation of miR-132 and miR-185 genes

As shown in Table 4, promoter methylation frequency of miR-132 and miR-185 genes showed no significant difference between cases with congenital factor XIII deficiency and healthy controls. The analysis of OR detected no significantly increased risk of disease for homomethylated.

21

Table 4 . The socio-demographic characteristics of the case and control groups in miR-185 and miR-133.

Characteristic		Controls (N = 74)	MiR-185 methylation			MiR-132 methylation			Cases (N = 75) Present	MiR-185 methylation			MiR-132 methylation		
			M	U	MU	M	U	MU		M	U	MU	M	U	MU
Sex	male	36 ± 0/502	-	3 (8.3)	33 (91.6)	3 (8.33)	3 (8.33)	30 (83.33)	34 ± 0/503	-	1 (2.9)	33 (97.05)	1 (2.94)	0	33 (97.05)
	female	38 ± 0/058	2 (5.2)	5 (13.15)	31 (81.578)	1 (2.63)	-	37 (97.36)	41 ± 0/058	3 (7.31)	-	38 (92.68)	0	0	41 (100)
Family History	Yes	-	-	-	-	-	-	-	47	3 (6.38)	-	44 (93.61)	0	0	47 (100)
	No	-	-	-	-	-	-	-	28	-	1 (3.57)	27 (96.42)	1(3.57)	0	27 (96.4)
Intracranial bleeding	Yes	-	-	-	-	-	-	-	17	1 (5.88)	1 (5.88)	15 (88.23)	0	0	17 (100)
	No								58	2 (3.45)	-	56 (96.55)	1(1.7)	0	57 (98.27)

Table 5. Comparison of relative gene expression for Micro132 and Micro185 genes between in patients with congenital factor XIII deficiency and healthy controls.

Genes		Sex		p-Value	Age	p-Value	No.	Mean±SD	p-Value
		Male	Female						
Micro132	Cases	5	10	0.052	13.4		15	0.92±0.13	
	Controls	7	8		13.7	0.25	15	0.99±0.07	0.126
Micro185	Cases	5	10	0.052	13.4	0.25	15	0.93±0.1	
	Controls	7	8		13.7		15	0.98±0.1	0.165

Heteromethylated patterns of miR-132and miR-185 in comparison to the unmethylated pattern as reference [OR = 0, 95% confidential 0 - 65, *p* = 0.78 .OR = 4.41, 95% confidential 0.42 - 220.4, *p* = 0.33, OR = 0.08, 95% confidential 0.001 - 2.05, *p* = 0.19, OR = 0.74   , 95% confidential 0.06 - 6.68, *p* = 0.9 respectively].

MiR-132 and miR-185 genes expression

Because of limitation for obtaining fresh sample only 15 patients in case group as well as 15 cases in control group during the study time were eligible for sampling and obtaining fresh sample for RNA extraction and subsequently for gene expression. Among case group, five and ten and in control group seven and eight persons were male and female respectively and this difference was not statistically significant (*p*: 0.052).

Expression an analysis revealed no statistically significant difference between cases and healthy controls concerning the relative expression of miR-132 and miR-185 (Table 5).

Discussion

FXIII, a protransglutaminase distributed in blood plasma and platelets, is involved in clot preservation by converting loose fibrin polymers into an organized structure by cross linking the peptide-bound glutamyl and lysine residues of fibrinogen chains through an isopeptide bond. Therefore, it participates in physiologic processes including wound repair and healing (Fadoo et al., 2013). Unlike plasma FXIII, which is in the form of a tetrameric structure (A2B2), the cellular form of FXIII present in platelets, monocytes and tissue

macrophages is a homodimer of FXIII-A subunit (Hsieh and Nugent, 2008). FXIII-A is synthesized by megakaryocytes and packaged into budding platelets, and it is present in large amounts in circulating forms. It seems to play an important role in the cytoskeletal remodelling associated with activation stages of platelets. FXIII-A is also found in blood monocytes and in all subsets of monocyte-derived macrophages in every part of the body. FXIII-A is mainly localized in the cytoplasm in association with cytoskeletal filaments; however, in a relatively early stage of macrophage differentiation, it appears temporarily in the nucleus. Cytoplasmic expression has a very close relationship with phagocytic activities (Adany et al., 2003). mRNA 3´UTRs of FXIII-A has binding sites for multiple individual miRNAs, which acts as posttranscriptional regulators of gene expression. Srikanth Nagalla et al (2011) demonstrated that miRNAs are able to repress expression of platelet proteins (Nagalla, 2011). Emerging evidences now support the idea that DNA methylation is crucially involved in dysregulation of miRNAs in different type of disorders. Shilpa Jain et al (2013) showed that platelets from sickle cell anemia patients exhibit an altered miRNA expression profile (Jain et al., 2013). Michael Girardot et al (2010) found miR-28 overexpressed in platelets of a fraction of myeloproliferative neoplasm patients, while it was expressed at constantly low levels in platelets from healthy subjects (Girardot et al., 2010). Lu Qian Wang (2013) showed that in miR-9 family, miR-9-3 is a relatively frequently methylated tumor suppressor miRNA, and hence silenced in chronic lymphocytic leukemia (CLL), while miR-9-1 methylation is rare in CLL (Wang et al., 2013). Pinto R et al (2013) showed different methylation and microRNA expression patterns in male and female familial breast cancer patients (Pinto et al., 2013). Tao Huang et al (2012) detected dysfunctions associated with MiRNA expression in lung cancer (Huang et al., 2012). From a therapeutic standpoint, however, manipulating the expression level of a specific miRNA may have only a limited effect on expression of the desired target gene, but targeting a single 3′UTR by multiple miRNAs can result in a synergistic mode of regulation. Improved understanding of miRNA epigenetics might lead to the development of a new generation of diagnostic markers for congenital factor XIII deficiency (Thomson et al., 2011; Richardson and Patel, 2014) .A limitation related to the samples was that due to timing and problems associated with fresh sample collection, the same samples were not used in methylation and gene

expression studies. The present study results suggest that further research is necessary in this field to find an exact epigenetic change during diagnosis and treatment of congenital factor XIII deficiency in the same blood sample, which can be used as a fast and simple marker to monitor the disease process.

Acknowledgment

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Open Access Statement:

A Long Noncoding RNA, ANCR, Is Upregulated in Bladder and Breast Tumor Tissues

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Abstract

Long non-coding RNAs (lncRNAs) have recently found to have important regulatory roles, and their aberrant expressions and functions are directly linked to carcinogenesis. Both urinary bladder and breast tumors are prevalent neoplasms, with high rates of incidence. To identify a potential expression alteration of the recently discovered "anti-differentiation non-coding RNA, (ANCR), during tumorigenesis, we initially assessed its expression in several cancer cell lines (LNCAP, MCF-7, Ht-29, 5637, A549, HepG2, and PC3) and then compared its expression variability in tumor vs. non-tumor samples of bladder and breast. Here, ANCR expression profile was studied by qRT-PCR in paired tumor and marginal non-tumor samples obtained from patients that had been referred to the Labbafi-Nejad and Imam Khomeini Hospitals, respectively. Our data revealed a significant upregulation ($p = 0.003$) of ANCR in breast tumor tissues, in comparison to non-tumor marginal specimens from same patients. Similar upregulation was also detected in bladder tumor samples, however, this alteration was not statistically significant ($p \geq 0.05$), probably due to small number of samples (n = 10). In conclusion, our results suggest a possible role of ANCR in tumorigenesis of bladder and breast tissues, as well as its potential usefulness as a novel diagnostic biomarker for bladder and breast tumors.

Keywords: lncRNA, ANCR, Breast Cancer, Bladder Cancer

Introduction

Long non-coding RNAs, > 200 nucleotide- non coding RNAs, are transcribed extensively throughout the genome, and regulate various life processes including development, differentiation, and pluripotency. They regulate these processes mostly by fine tuning gene expression, and cellular mechanisms such as chromatin modification, transcription and posttranscriptional processes (Gabory et al., 2010; Gupta et al., 2010; Mercer et al., 2009; Tripathi et al., 2010). Due to these functions, lncRNAs are emerging as critical players in tumorigenesis and several studies have reported lncRNAs deregulation in a number of cancers (Congrains et al., 2013; Huarte et al., 2010; Huarte and Rinn, 2010; Nakano et al., 2006; Redon et al., 2010). Such studies have demonstrated that this class of the non-coding RNAs contains molecules with both oncogenic and tumor suppressive

functions, suggesting that their aberrant expressions may be a contributor in carcinogenesis. Urinary bladder cancer (UBC) is the 9th most prevalent cancer around the world (Murta-Nascimento et al., 2007), with the rate of incidence in males being much higher than in females. Annually about 261000 new cases are diagnosed with UBC, and roughly 115000 patients die from it, mostly due to a late diagnosis (Christoforidou et al., 2013; Larsson et al., 2008). The difference between the incidence and mortality rates suggests that UBC has a long progression period (Murta-Nascimento et al., 2007). UBC is mostly common in developed countries (Murta-Nascimento et al., 2007), while in Iran the rate of incidence is moderate accounting to <8.8/100000 in males and < 2.8/ 100000 in females (Parkin et al., 2001).

Breast cancer is also the most prevalent cancer among women and ranks the 2nd, after lung cancer. However, due to a relatively good prognostic procedure, it ranks fifth in the list of cancer-caused death (Parkin et al., 2001; Sharif et al., 2010).

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In Iran breast cancer is also the most common cancer among women, comprising about 21.4% of all cancers affecting female population (Noroozi et al., 2011). Moreover, it has been shown that in Iran younger women are at relatively higher risk to preventative and therapeutic strategies to combat breast develop breast cancer than Western women (Harirchi et al., 2000). Therefore, in Iran more advance malignancies needed to be developed.

Anti-differentiation non-coding RNA, (ANCR), is a lncRNA gene located on human chromosome 4 upstream of USP46 gene, and embeds MIR4449 and SNORNA26 within its 1st and 2nd introns. Although these small non-coding RNAs are co-expressed with ANCR, but they are not part of the mature lncRNA transcript. ANCR gene encodes a single 855-nucleotide transcript with diminished expression in different terminally differentiated cell types (keratinocytes, adipocytes and osteoblasts), suggesting that it controls differentiation genes scattered throughout the genome (Kretz et al., 2012; Zhu and Xu, 2013).

In this study, we initially assessed the expression of ANCR in various tumor cell lines, and then examined a potential gene expression alteration of this lncRNA in urinary bladder and breast tumor and non-tumor samples.

Materials and Methods

Cultivation of human cell lines

Cell lines originated from bladder carcinoma (5637), breast adenocarcinoma (MCF-7), hepatocellular carcinoma (HepG2), prostate cancer (PC3), prostatic adenocarcinoma (LNCAP), lung adenocarcinoma (A549), and human colorectal adenocarcinoma (Ht29) were obtained from Pasteur Institute of Iran. They were cultured at 37ºC with 5% humidified CO2 in RPMI or high glucose DMEM medium (Invitrogen, UK) supplemented with 10% FBS (Invitrogen, UK), 100 U/mL penicillin, 100 mg/mL streptomycin, and 25 ng/mL amphotericin B.

Clinical Samples

Urinary bladder tissue samples from cancer patients had been underwent surgery in Labafi-nejad hospitals, were collected whereas breast tissue samples were provided by Iran bank of tumor tissues, in Imam Khomeini hospital. Tissues were immediately snap frozen using nitrogen before extracting their total RNA. In addition, the whole procedure was approved by the ethics committees of Tarbiat Modares University and Labafi-nejad hospital. Patients' written informed consents were also collected prior to sampling. Pathological parameters for tumor grading were evaluated according to the WHO criteria.

RNA Extraction and cDNA synthesis

Total RNA was extracted from both bladder and breast tissues as well as from tumor cell lines, using TRIzol reagent (Invitrogen, USA), according to the manufacturer's instructions. To evaluate the purity and integrity of the isolated RNA, RNA samples were separated by agarose gel electrophoresis and their concentration was measured by optical absorbance at 260 nm. Furthermore, 4 µg of the extracted RNA were treated by RNase-free DNaseI (Takara, Japan) to remove any trace of DNA contamination. Then, 1 µg of DNase-treated RNA was used for Reverse transcription by employing PrimeScript TM Reagent kit (Takara, Japan).

Quantitative Real-time PCR and regular RT-PCR

The specific primers for regular and quantitative RT-PCR to amplify both ANCR (GenBank accession numbers NR_024031.1) and β2 microglubolin (β2M, as an internal control; GenBank accession number NM_004048.2) were designed using Gene runner (version 3.02; Hastings Software), PerlPrimer v1.1.16, and Oligo v 6.54 softwares.

Regular RT-PCR reactions were performed using 10 µl of Taq DNA polymerase master mix RED (Ampliqon, Denmark), 2µl of synthesized cDNA, and 0.5 mM of each primer. PCR cycles were performed using the following conditions: initial denaturation at 94ºC for 5 min, followed by 30 and 25 cycles (for ANCR and β2M amplification, respectively) of 94ºC for 35 seconds, 60ºC for 30 seconds, and 72ºC for 35 seconds, with a final extension at 72ºC for 10 minutes. The aforementioned primers amplified a 472 bp fragment of ANCR and a 167 bp fragment of β2M. Then, the PCR products were separated on a 1.5% agarose gel, stained with ethidium bromide and visualized under UV light. The validity of PCR products was confirmed by direct DNA sequencing (Microgen, South Korea). The sequences of the forward and reverse primers were shown in table 1. 2 µl of cDNA, 10 µl of SYBR-Green ready mix, and 0.1 µl of Rox, and 0.5 µM of each specific primer were used to carry out quantitative PCR (q-PCR). β2M gene was used as an internal control, and the expression of other genes were normalized to its expression level.

Table 1. Primer Sequences

Primer name	Sequence
β2MF	5-GGGTTTCATCCATCCGACATTG-3
β2MR	5-TGGTTCACACGGCAGGCATAC-3
ANCR-F1	5-GCCACTATGTAGCGGGTTTC-3
ANCR-R1	5-CAGAGTATTCAGGGTAAGGGTC-3
ANCR-R2	5-GTAGTTGTCAACCTGCGCTAAG-3

ABI 7500 real-time PCR system (applied Biosystems, Foster city, CA) was used to perform the PCR using the following cycling conditions: initiation at 95°C for 15 minutes, amplification for 40 cycles with: denaturation at 95°C for 15 seconds, annealing at 62.5^{o}C for 30 seconds, and extension at 72°C for 30 seconds. To validate the PCR products, melt curves were analyzed, and amplified products were sequenced.

Statistical Analysis

The relative expression values were calculated by using the ΔΔCT method, by normalization to the internal control β2M, and those of non-tumor samples in both breast and bladder tissues, both used as calibrators to calculate the fold change of relative expression. The significance of difference in the gene expression levels was determined by student's t-test using GraphPad Prism 6.0. Furthermore, all qPCR experiments were carried out in duplicates.

Results

Two sets of primers were designed to delineate the expression patterns of ANCR in various cell lines, urinary bladder tumors, and breast tumors (Figure 1). The designed primers serve to amplify a 472 bp (using F1 and R1 primers) and a 171 bp (F1 and R2 primers) fragment of ANCR cDNA by regular RT-PCR or quantitative RT-PCR, respectively. The authenticity of the PCR products was further confirmed by DNA sequencing. Moreover, DNase treatments as well as no-RT reactions, as negative controls, were employed to avoid genomic's DNA amplification.
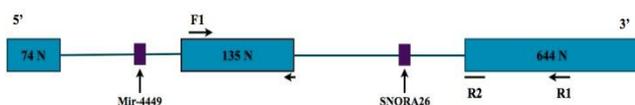

Figure 1. Genomic organization of ANCR. Two pairs of primers were designed; F1/R1 primers employed in regular RT-PCR and F1/ R2 primers used in qRT-PCR.

ANCR expression was delineated in seven different cell lines (5637, MCF7, HEPG2, PC3, A549, and Ht-29), and all of the examined cell lines were found highly expressing ANCR (Figure 2).
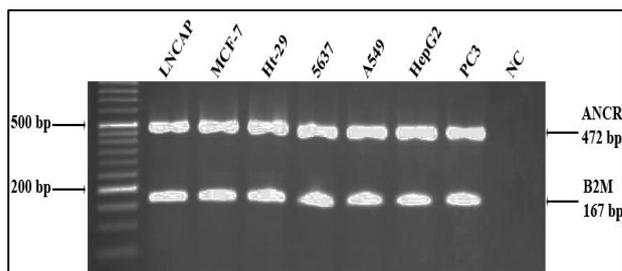

Figure 2. : Expression of ANCR in 7 different cancer cell lines. ANCR expression was detected in LNCAP, MCF-7, Ht-29, 5637, A549, HepG2, and PC3 cell lines. B2M gene was used as an endogenous control, and No-RT control (NC) sample was employed to validate the accuracy of RT-PCR products.

Moreover, the primers for conventional RT-PCR were designed in a way to permits the detection of any possible novel variants of ANCR. However, it failed to recognize and alternative spliced form for ANCR.

Additionally, the expression profile of ANCR was analyzed in 10 pairs of breast tumor and non-tumor samples, as well as 10 pairs of urinary bladder tumor/non-tumor surgical specimens. Initially, ANCR expression was normalized to that of endogenous control, β2M. Moreover, the non-tumor samples were used as calibrators to calculate the relative expression of ANCR. Based on our real-time data, ANCR found to have a higher expression level ($p = 0.003$; Figure 3A) in tumor samples, compared to that of apparently normal tissues obtained from the margin of same patients. Similar observation was made on upregulation of ANCR in bladder tumor samples, compared to their paired non-tumor specimens. However, the latter overexpression was not statistically significant ($p = 0.3$; Figure 3B).

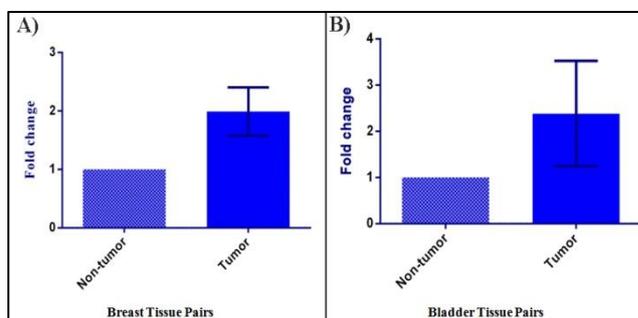**Figure 3:** ANCR expression in breast and bladder tumor and non-tumor tissue pairs. A) A significant ($p = 0.003$) upregulation in the expression of ANCR was detected in breast tumor specimens, in comparison to the non-tumor marginal samples obtained from the same patients. B) Similarly, in bladder samples ANCR was upregulated in tumor samples, but such alteration was not statistically significant ($p > 0.05$).

Discussion

In recent years, numerous reports have identified several lncRNAs with important regulatory parts in initiation and progression of cancers (He et al., 2014; Huarte and Rinn, 2010; Shahryari et al., 2014; Tian et al., 2014; Xiang et al., 2014). Such non-coding RNAs play crucial roles in almost every aspect of cell biology, from epigenetics modification to posttranscriptional regulation and splicing (Ip and Nakagawa, 2012; Mercer et al., 2009; Soreq et al., 2014; Yang et al., 2011; Zhou et al., 2014). These processes are linked directly to the genetics of cancer initiation and development. Therefore, aberrant expression of lncRNAs is expected to be a causative event of tumorigenesis in different tissues.

In the light of available statistics, both breast and urinary bladder cancers are associated with high incidence and mortality rates, therefore finding novel diagnostic and prognostic biomarkers, as well as to develop more efficient therapeutic strategies are needed. Accordingly, several researches have recently focused on misregulation of lncRNAs in bladder and breast cancers, and proposed such transcripts as potential diagnostic, prognostic, and therapeutic biomarkers (Crea et al., 2014; Lin et al., 2014; Shahryari et al., 2014; Zhang and Leung, 2014).

Considering the reports linking perturbations of lncRNAs and cancer, we aimed here to explore a possible link between ANCR expression and tumorigenesis in both breast and urinary bladder tumors. ANCR was reported to be downregulated in terminally differentiated keratinocytes and osteoblasts (Kretz et al., 2012; Zhu and Xu, 2013). These findings raised the possibility that this transcript has a critical function in the transition from progenitor to differentiated state of the cell. To the best of our knowledge, this is the first report to delineate the expression of ANCR in breast cancer and UBC.

Similar to HOTAIR, a lncRNA that interacts with Polycomb Repressive Complex 2 (PRC2) to regulate the chromatin state and found to be overexpressed in breast cancer (Gupta et al., 2010; Wu et al., 2013), our data showed that ANCR was also significantly upregulated in breast tumor tissues in comparison with the non-tumor control samples. On the other hand, in bladder cancers, Urothelial Cancer Associated-1 (UCA1) is highly expressed in embryonic tissues, bladder and other cancers, but not in adult tissues or adjacent non-tumor tissues, indicating that UCA1 may be involved in both embryonic development and tumorigenesis (Wang et al., 2008). Likewise, ANCR showed an overexpression in the urinary bladder tumor samples, but such increase was not statistically significant, probably due to a low number (10 pairs) of samples. These findings may highlight a strong correlation between the aberration in ANCR expression and tumorigenesis. Nevertheless, these data are preliminary and need to be validated by using more tissue samples. Therefore, with employing more samples, subdivided in different grades and stages of malignancies, we could better analyze the suitability of ANCR as a potential diagnostic and prognostic biomarker.

In this study, we also delineated the expression of ANCR in different cell lines originating from various tissues (5637, MCF-7, HepG2, PC3, A549 and Ht-29). The fact that ANCR is highly expressed in these cell lines suggests that it has probably a general causative role in other types of cancers.

In Conclusion, we depicted the expression pattern of ANCR in both urinary bladder and breast tumors. Our data is the first report on distinct expression of ANCR in breast and bladder cancers, demonstrating a possible link between the expression level of ANCR and tumorigenesis. Moreover, with further validation, ANCR can potentially be considered as a novel tumor biomarker with potential diagnostic, prognostic and therapeutic value.

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Renin-Angiotensin A1166C Polymorphism and the Rrisk of Stroke

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Abstract

Stroke is the leading cause of death and disability in the world after the cancer and cardiovascular diseases. Genetic factors have main significance to got stroke. Renin-angiotensin system contains candidate genes and polymorphisms for causing stroke. There are reported associations between stroke and angiotensin II type-1 receptor g. 1166A > C polymorphism (rs5186). Therefore in this study this association was investigated for the east Iranian population. This study is based on 201 stroke patients and 220 controls. To predict the genetic risk of stroke allele and genotype frequencies of angiotensin II type-1 receptor rs5186 were analyzed in this population according to stroke subtypes, gender, age, hypertension, diabetes mellitus, high and low density lipoprotein and triglycerides. According to statistical analysis no significant difference was found between case and control groups. But there were a significant relevance between total cholesterol and stroke ($p = 0.037$). In this population angiotensin II type-1 receptor g. 1166A > C polymorphism did not increase the risk of stroke. The main reason for this study is complex nature of gene-environment interactions in the pathophysiology of this disease.

Keywords: Stroke, Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP), Angiotensin II type-1 Receptor g. 1166A > C Polymorphism.

Introduction

Stroke has recognized as a multifactorial neurological disease, and is one of the most important causes of death and disability throughout the world (Deb Sharma and Hassan, 2010; Meschia et al., 2011). The question is that what is the molecular function of Stroke? And why people affected more and more with this disease. Each year 795000 people were affected a new or recurrent with stroke (Roger et al., 2011). Renin-angiotensin system (RAS) contains candidate genes for causing stroke (Hassan and Markus, 2000). This system is one of the most important physiological pathways that play a role in the maintenance of blood pressure (Harrison-Bernard, 2009). Angiotensin plays an important role in RAS pathway.

Angiotensin I convert to angiotensin II by angiotensin converting enzyme (ACE).

At least there are two main receptors for angiotensin II: angiotensin II type I receptor (AGTR1 or AT1R) and angiotensin II type II receptor (AGTR2). Angiotensin II is a primary regulator of aldosterone secretion and acts as a vasoconstrictor by binding to angiotensin II type 1 receptor (Fyhrquist and Saijonmaa, 2008; Kobori et al., 2007). AT1R location is 3q23-25 as well as it contains more than 55 kb length and 5 exons between 59 to 2014 bp size ranges. First four exons encode a 5´untranslated region (Abdollahi et al., 2005; Guo et al., 1994). The activated receptor couples to G-protein and thus effect on intracellular messengers including phospholipase C, Ca^{2+} and protein kinase C (Carey and Siragy, 2003). There are lot polymorphisms in RAS pathway genes' (Gargano et al., 2009; Rupert et al., 2003; Wong et al., 2008). During the last years, there has been

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considerable debates over the association of angiotensin II type-1 receptor g. 1166A > C polymorphism and risk of stroke, myocardial infarction and hypertension (Brenner et al, 2005; Hahntow et al., 2010; Léon H Henskens et al., 2007; Lapierre et al., 2006; Rubattu et al., 2004; Takami et al., 2000). This polymorphism has been considered as a risk factor for stroke in several populations (Agachan al., 2003; Möllsten, Stegmayr et al., 2008; Szolnoki et al., 2006; Takami et al., 2000). In contrary, other studies have not pose AT1R 1166A > C as a risk factor for stroke (Hindorff et al., 2002; Zhang et al., 2010; Zhao et al., 2001).

Stroke incidence in Iran is considerably great than most western countries (Azarpazhooh et al., 2010). In this regard, in the present study a population based case-control study has been used to prospectively investigate the association of AT1R/1166A > C and stroke in east Iranian population.

Materials and Methods

Study population

In this study 201 randomly subjects were selected at the Ghaem hospital between March 2012 and December 2013 according to the following criteria: clinical symptoms of a stroke based on world health organization definition for stroke and ages between 20 and 70. In the cases group of this case-control study there were 86 males and 115 females. Stroke subtypes in subjects were determined by experienced neurologist according to the TOAST (Trial of ORG 10172 in Acute Stroke Treatment). To determine the type of stroke computed tomography (CT) scan and magnetic resonance imaging (MRI) was used. In the control group there were 96 males and 124 females (220 controls) without any history and clinical evidence of cerebrovascular disease. In control and stroke patient groups biochemical analysis were measured. Both groups were matched in age, sex. Stroke risk factors containing hypertension, diabetes, ischemic heart disease (IHD), low density lipoprotein (LDL), high density lipoprotein (HDL), triglyceride and cholesterol was analyzed. Patients by fasting blood glucose $\geq$ 126 mg/dl were diagnosed as diabetes mellitus. People with Hypertension were determined in Systolic blood pressure (SBP) $\geq$ 140 mmHg or diastolic blood pressure (SBP) $\geq$ 140 mmHg.

DNA Extraction

Venus blood was collected in tube containing ethylene diamine tetra acetic acid (EDTA). DNA was extracted from 200 µl of blood samples by PrimePrep Genomic DNA isolation kit from blood (catalog No K-2000; Genet Bio) and checked by 1% agarose gel. Blood samples were stored at -20ºC.

Genotyping

The AT1R/1166A > C polymorphism was identified using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP). The forward primer was 5´-AAAAGCCAAATCCCAC TCAA and the reverse primer was 5´-CAG GACAAAAGCAGGCTAGG (21). PCR was carried out with an Applied Biosystems 2720 thermal cycler. PCR amplification was performed in 25 µl reaction volume containing, 0.5 µl of DNA (40-80 ng), 0.4 µM of each primer, 0.25 mM of dNTPs, 1.5 mM $MgCl_2$ and 0.5 U *Taq* DNA polymerase. The PCR amplification conditions were as follows: initial denaturation at 96 ºC for 120s, followed by 35 cycles of 30s at 96 ºC, 30s annealing at 53 ºC, extension for 60s at 72ºC and final extension for 10 minutes at 72 ºC. PCR products with 432bp length were analyzed on 2% agarose gel. PCR products were digested by DdeI restriction endonuclease at 60 ºC overnight. Restriction fragment products were 58 and 374 bp for A allele and 58, 143 and 231 bp for C allele. Products were detected by electrophoresis on 3% agarose gel stained with ethidium bromide (Firure 1).
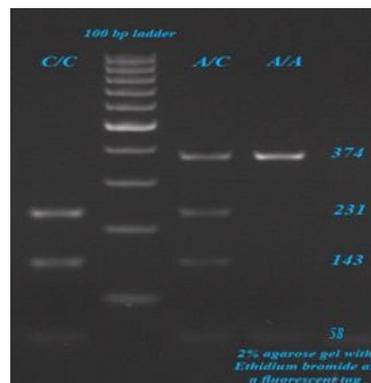
Figure 1. Restriction fragment products. 58 and 374 bp for A allele and 58, 143 and 231 bp for C allele.

Statistical analysis

The normality of numeric variables was assessed using Kolmogorov-Smirnov test and Deviation from Hardy-Weinberg equilibrium was tested by χ^2 test. Quantitative data were compared by Student's t-test and qualitative data such as genotypes and alleles were analyzed by the χ^2 and Fisher's exact test. Allele frequencies and genotype distribution

between case and control groups were compared by χ^2 test. Each stroke subtype was compared with their matched control subjects. Stroke risk factors were analyzed between both case and control groups. Two tailed pvalue of 0.05 was considered for data analyses.

Results

The demographic and clinical characteristics of the study population are shown in table 1.

Table 1. Clinical characteristics of study participants

Characteristics	Cases	Controls	*p*-value
Age (mean ± SD)	51.5 ± 13.8	50.3 ± 12.2	0.169
Sex (male/Female)	86/115	96/124	0.860
Diabetes mellitus (n, %)	57 (28.4)	45 (20.5)	0.055
LDL	127.0 ± 34.5	121.1 ± 32.1	0.094
HDL	40.8 ± 9.5	42.2±8.6	0.161
Triglyceride	140.2 ± 93.1	142.3 ± 86.6	0.796
Total Cholesterol	185.3 ± 45.9	190.3 ± 42.2	0.283

Stroke subtypes distribution in case group was: Ischemic 111 (55.2%), hemorrhagic 66 (32.8%) and other subtypes 24 (11.9%). Risk factors for stroke such as hypertension, diabetes mellitus, total cholesterol, high and low density lipoprotein and triglycerides were analyzed between case and control groups and there were no significant different between them ($p > 0.05$). The Hardy-Weinberg equilibrium was assessed in patient and control groups and both allele and genotype distribution was in accordance with it ($p > 0.05$). Alleles and genotypes frequency between stroke and control groups are presented in table 2, 3 and 4. There was no association between case and control groups in AT1R/1166A > C genotypes or allelic distribution.

Table 2. Alleles and genotypes frequency for male

Genotype and alleles	Male		*p*-value
	Stroke N= 87	Control N= 96	
AA n (%)	57 (65.5)	67 (69.8)	0.501
AC n (%)	27 (31.0)	25 (26.0)	0.425
CC n (%)	3 (3.4)	4 (4.2)	0.812
A n(%)	140 (80.9)	159 (82.8)	0.03
C n (%)	33 (19.1)	33 (17.2)	0.860

Table3. Alleles and genotypes frequency for female

Genotype and alleles	Female		*p*-value
	Stroke N=1 14	Control N= 124	
AA n(%)	76 (66.7)	84 (67.7)	0.897
AC n (%)	38 (33.3)	35 (28.2)	0.419
CC n(%)	0 (0.0)	5 (4)	0.03
A n (%)	190 (83.3)	203 (81.9)	0.8
C n (%)	38 (16.7)	45 (18.1)	0.537

Table 4. Total alleles and genotypes frequency

Genotype and alleles	Total		*p*-value
	Stroke N= 201	Control N= 220	
AA n (%)	133 (66.2)	151 (68.6)	0.589
AC n (%)	65 (32.2)	60 (27.3)	0.256
CC n (%)	3 (1.5)	9 (4.1)	0.110
A n (%)	331 (82.3)	362 (82.3)	0.110
C n (%)	71 (17.7)	78 (17.7)	0.589

In addition, the analysis was done in the subtypes of stroke, age, sex and other risk factors but except in one case there were no significant difference among patients and controls.

Discussion

In the present study the genotypic and allelic frequency of AT1R/1166A > C polymorphism in stroke patients and controls were examined. Neither genotype distribution nor the allelic frequency differed significantly between the case and control groups. Further subgroup analysis including stroke subtypes, gender, age, hypertension, diabetes mellitus, high and low density lipoprotein and triglycerides showed any direct association. Renin-angiotensin system plays a major role in blood pressure which is one of the most effective factors in stroke development. There are several polymorphisms in this pathway which all of them can be potentially a risk factor for stroke (Jia et al., 2014; Tsai et al., 2014). Among them AT1R/1166A > C is a candidate SNP which previously studied in different populations. A nested case-control study on 257 northern Sweden subjects who suffered a first ever stroke and 549 controls demonstrated AA genotype can increase risk of stroke (Möllsten et al., 2008). Henskens et al. have determined AGTR1 A1166C polymorphism is in a significant association with Silent white matter lesions (WMLs), as lesion volume was lowest in the

presence of an AGTR1 C allele and CC genotype (Léon HG Henskens et al., 2005). Rubattu et al. study supports the role of AT1R/1166A > C polymorphism in the development of ischemic stroke among Sardinia population. They assessed 215 cases and 236 controls in this population (Rubattu et al., 2004). In contrary, some other study demonstrated no association between this SNP and stroke. Szolonki et al. study on 308 patients and 272 neuroimaging alteration-free subjects showed AT1R/1166A > C polymorphism cannot be considered as a risk factor for stroke (Szolnoki et al., 2006). In a study of 800 African Americans and 1371 whites reported that this SNP is not associated with stroke (Hindorff et al., 2002). In a meta-analysis which has been performed by zhang et al. no significant association was found between A1166C polymorphism and ischemic stroke in Asian population (Zhang et al., 2010). In a nut shell, there are several studies demonstrating significant association between AT1R/1166A > C polymorphism and stroke but this study performed that this SNP cannot be considered as an independent risk factor for stroke in this population. It could be because of Iranian particular genetic context. It is worth nothing that, the biological relevance of the angiotensin II type-1 receptor g. 1166C polymorphism is unclear and further well-designed studies are needed to identify the biological cause of this relationship between angiotensin II type-1 receptor g. 1166C polymorphism and stroke.

Acknowledgment

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Open Access Statement:

Application of Random Amplified Polymorphic DNA (RAPD) to Detect the Genotoxic Effect of Cadmium on Tow Iranian Ecotypes of cumin (*Cuminum cyminum*)

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Abstract

Cadmium, a metal widely used in industrial processes, has been recognized to be a highly toxic and dangerous environmental pollutant. Random amplified polymorphic DNA (RAPD) test is a feasible method to evaluate the toxicity of environmental pollutants on vegetal organisms. Herein, two Iranian ecotypes of *Cuminum cyminum* (cumin) plantlets following exposure to cadmium (Cd) concentrations of 300–1050 µM for 7 days were screened for DNA genetic alterations by DNA fingerprinting. 10 RAPD primers of 50–70% GC content were found to produce unique polymorphic band profiles on treating cumin seedlings. After Cd treatment, significant changes were observed in RAPD profiles of both ecotypes. These changes included variation in band intensity, disappearance of bands, and appearance of new PCR products in comparison to the control group, and they were dose dependent. These results indicated that genomic template stability (GTS, a qualitative measure reflecting changes in RAPD profiles) was significantly affected at the above Cd concentrations. The GTS index in both ecotypes gradually decreased with an increase in Cd concentrations. These findings suggest that DNA polymorphism detected by RAPD analysis could be a powerful eco-toxicological tool to evaluate the genotoxic effects of cadmium on plants.

Introduction

Heavy metal toxicity is one of the major current environmental health problems and potentially dangerous due to bioaccumulation through the food chain and in plant products for human consumption. Therefore, heavy metal contaminations of soils and plants have become an increasing problem (Michael et al., 2012).

Cadmium (Cd) is a highly toxic heavy metal that enters the environment mainly from industrial processes and phosphate fertilizers. It can reach high levels in agricultural soil and easily accumulates in plants. Cd ions are taken up readily by the plant roots and translocated to the above-ground vegetative parts (Shamsi et al., 2008).

The presence of Cd at higher concentrations in the soil damages root tips, reduces nutrient and water uptake, impairs photosynthesis and inhibits growth of the plants (Sharma and Dubey, 2006). Furthermore, Cd directly or indirectly induces reactive oxygen species (ROS), which affect the redox status of the cell and cause oxidative damage to proteins, lipids, and other biomolecules (Schutzendubel et al., 2001; Amirthalingam et al., 2013). Cd damages the nucleoli in cells of root tip, alters the synthesis of RNA, inhibits ribonuclease activity and inhibits the DNA repair mechanism (Amirthalingam et al., 2013).

The genetic material (DNA, except in the case of some viruses in which it is RNA) carries the information necessary for determining the properties of an organism and is responsible for transmitting information from parent to offspring. During the cell cycle, DNA undergoes conformational and structural changes, but it is of paramount importance that its structural integrity be maintained throughout the life of the cell. Organisms are continuously exposed to environmental stresses that may result in DNA damage. Genotoxicity of metals such as Cd is directly related to its effect on the structure and function of DNA and several studies have used the comet assay, micronucleus assay or chromosome aberration assay to measure the genotoxic effect of metals on plants (Angelis et al., 2000; Liu et al., 2009a,b; Cambier et al., 2010). Advantages of measuring effects of genotoxic chemicals directly

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on DNA are mainly related to the sensitivity and short response time. Recently, the development of molecular marker technology has provided new tools for detection of genetic alteration in response to heavy metal tolerance by looking directly at the level of DNA sequence and structure. The random amplified polymorphic DNA (RAPD) assay, developed by Williams et al. (1990) and Welsh and McClelland (1990), is a PCR-based technique that amplifies random DNA fragments of genomic DNA with single short primers of the arbitrary nucleotide sequence under low annealing conditions.

This technique is used extensively for species classification, genetic mapping and phylogeny etc. In addition, their use in surveying genomic DNA for evidence of various types of DNA damage and mutation shows that RAPD may potentially form the basis of novel biomarker assays for the detection of DNA damage and mutational events (e.g. rearrangements, point mutation, small insertions or deletions of DNA and ploidy changes) in cells of bacteria, plants, invertebrate and vertebrate animals (Savva, 1996; Savva, 2000; Atienzar et al., 2000).

Fragments generated by RAPD are visualized after agarose gel electrophoresis and ethidium bromide staining. The resulting DNA profiles may differ due to band shifts, missing bands or the appearance of new bands. Detection of genotoxic effect using RAPD involves the comparison of profiles generated from control (unexposed) and treated (exposed) DNA.

The aims of this study was the evaluation of the random amplified polymorphic DNA (RAPD) assay for the detection of DNA damage in two Iranian ecotypes of cumin (*C. cyminum*) seedlings measured in laboratory condition.

Materials and Methods

Plant materials and growth condition

The experimental plant used in this investigation was two Iranian ecotypes of cumin (*C. cyminum*) seeds, namely Isfahan and Khorasan that kindly provided by Dr. Mahammadinejad, Department of Agronomy and Plant Breeding, Shahid Bahonar University of Kerman, IRAN. Seeds were surface sterilized by immersion in 70% ethanol for 3 min., followed by stirring in sodium hypochlorite (5% chlorine) for 20 min. The surface sterilized seeds were rinsed 3 times with sterile distilled water for 5 min. The seeds then placed in petri dishes (90-mm diameter) on filter paper (Whatman # 42) under dark condition, so simulate the soil condition at

20°C. Uniform germinated seeds were transferred to petri pots (diameter 9.5 cm and height 14 cm) containing sands at sizes 1-3 mm (40%) and perlite (60%). Petri pots were incubated in a growth chamber at a temperature of 20 ± 2°C and a 16-h-day-8-h-night photoperiod. The plants were fertilized by Hoagland solution once a day. After 21 days, seedlings were treated with 0 (as control), 300, 450, 600, 750 and 1050 µM of Cd under the above mentioned laboratory condition for 7 days.

DNA extraction and RAPD procedures

For both the control and Cd treatments, shoots were collected and flash frozen in liquid nitrogen prior to storage at -70°C. Total DNA was extracted from approximately 100 mg of shoots using a CTAB protocol (Doyle and Doyle, 1987). The quantity and quality of DNA were estimated using scan Drop UV-Visible spectrophotometer (Analytik Jena, Germany) at 260 nm and 280 nm. The integrity of DNA was examined by gel electrophoresis in 1% agarose in 0.5 X TBE buffer. Electrophoresis of DNA was performed at 100 V for 35 min and DNA was visualized with a UV Transilluminator after staining with ethidium bromide

PCR conditions were optimized according to Williams et al. (1990). PCR was performed in a reaction mixture of 25µl containing approximately 80 ng of genomic DNA, 20 pmol of decanucleotide primer (Table 1), 200 µM dNTPs (50 µM of each), 2 mM $MgCl_2$, 1 U of *Taq* DNA polymerase (Thermo Scientific) and 1X reaction buffer (10 mM Tris-HCl pH 8.3, 50 mM KCl).

For amplification, the reaction mixtures were denatured at 94°C for 3 min, followed by 35 cycles at 94°C for 30 sec (denaturation), 37°C for 60 sec (annealing) and 72°C for 60 sec (extension), with an additional extension period of 8 min at 72°C. All amplifications were conducted twice in order to evaluate the reproducibility of the polymorphic bands. A negative control, without genomic DNA, was run in parallel with every set of samples to confirm that no contaminating DNA was present in the reactions. Amplification was carried out in a thermocycler (Eppendorf, Germany) with heated lid. Amplification mixtures were stored at 4°C before use.

PCR reaction products were mixed with one-fifth volume of gel loading buffer (SinaClone, Iran), and then separated by electrophoresis on a 1.2% agarose gel, using a Tris-borate-EDTA (TBE) system (0.5 x TBE = 45 mM Tris-base, 45 mM boric acid, and 1 mM EDTA). Agarose gel dimensions were $10 \times 8 \times 0.5$ cm^3. Electrophoresis

was carried out at room temperature at 75 V for 1.5 h and after which the gels were stained with EB solution (0.015%) in distilled water. The image of the gel was visualized with a UV Transilluminator and the size of each amplification product was estimated using the Total Lab TL120 software. For comparison, DNA molecular size marker (100 bp DNA Ladder, Thermo Scientific) was used for each agarose gel. In all PCR-gels, the marker bands visualized were, from top to bottom, 3000, 2000, 1500, 1200, 1000, 900, 800, 700, 600, 500, 400, 300, 200 and 100 bp

Table1. Sequences of primers used in this study

Primer	Sequences of primer (5 → 3)	Primer	Sequences of primer (5 → 3)
OPE-07	AGATGCAGCC	OPI-14	TGACGGCGGT
OPF-05	CCGAATTCC C	OPJ-19	GGACACCACT
OPJ-01	CCCGGCATAA	OPQ-06	GAGCGCCTTG
OPJ-20	ACGAGGGACT	OPB-17	AGGGAACGAG
OPP-06	GTGGGCTGAC	OPS-17	TGGGGACCAC
OPU-20	ACAGCCCCCA	OPK-14	CCCGCTACAC
OPC-08	TGGACCGGTG	OPJ-21	ACGAGGGACT

Estimation of genomic template stability

Genomic template stability (GTS, %) was calculated as follows:

$$GTS\ (\%) = (1 - \frac{a}{n}) \times 100$$

Where *a* is the average number of polymorphic bands detected in each treated sample, and *n* is the number of total bands in the control. Polymorphism observed in RAPD profiles included the disappearance of a normal band and appearance of a new band in comparison to control RAPD profiles (Atienzar et al., 1999; Luceri et al., 2000), and the average was then calculated for each experimental group exposed to different Cd treatment.

Estimation of band sharing index

In our investigations, we have used the band sharing index (BSI) as a measure of similarity between two samples and this was calculated using the following equation:

$$BSI = 2s/\ (a + b)$$

Where *s* is the number of bands shared between two samples, *a* is the number of bands in the first sample and *b* the number of bands in the second sample. A BSI value of 1 indicates that two samples are identical, while a BSI value of zero indicates that two samples are totally different (Savva, 2000).

Results

DNA extraction

The suitability of the CTAB extraction method for DNA extraction was evaluated on the basis of the DNA purity and integrity. Concentration and purity of DNA extracted are usually measured at OD260 and by 260 nm/280 nm absorbance ratio. The purity grade of DNA extracted from the control and the exposed seedlings for 7 days of treatment was in the range of 1.6–1.7, and the yield obtained was approximately 85 μg (100 mg)$^{-1}$ (FW) tissue in the control plantlets. The integrity of the genomic DNA extracted by the CTAB method was shown in Figure 1. These results indicated that the CTAB method was suitable for DNA extraction from cumin and yielded high quality DNA samples, which is crucial for good RAPD analysis (Williams et al., 1990).
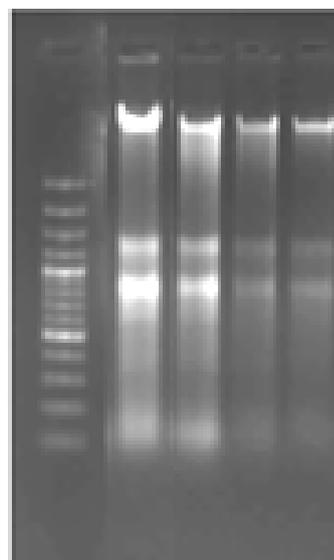

Figure 1. Genomic DNA of about 150-400 ng extracted by CTAB in *C. cyminum*

Effect of Cd stress on RAPD profile

In total, fourteen 10-mer oligonucleotide primers were utilized for screening the cumin genome for alteration in response to Cd stress, whilst only 10 priming oligonucleotides yielded specific and stable results (Figure 2 and Table 2). In all cases, RAPD patterns generated by the Cd-exposed plantlets were clearly different from those obtained using control DNA and exhibited a distinct change with increasing Cd concentration in both ecotypes. The differences in RAPD patterns refer to band intensity, loss of normal bands and appearance of new bands as compared with the control.

Table 2. Changes of total bands in control, and of polymorphic bands and varied bands in Cd-treated seedlings of Isfahan ecotype (up) and Khorasan ecotype (below).

No. of primers	Cd concentration (µM)																				
	0	300				450				600				750				1050			
		a	b	c	d	a	b	c	d	a	b	c	d	a	b	c	d	a	b	c	d
OPE-07	8	0	0	1	2	0	0	1	2	0	0	1	1	0	0	1	2	0	6	2	0
OPF-05	8	1	0	1	0	1	0	1	0	1	1	2	0	2	0	1	1	2	0	1	1
OPJ-01	6	2	0	2	4	2	0	2	5	2	0	2	5	2	0	2	6	2	2	0	5
OPJ-20	8	0	0	7	0	0	0	7	0	0	0	7	0	0	0	7	0	0	0	7	0
OPP-06	5	1	0	0	2	0	0	1	2	2	0	0	4	0	0	0	4	0	0	0	3
OPU-20	11	1	1	3	0	1	1	4	0	1	2	2	2	1	2	1	2	1	2	5	0
OPC-08	3	1	0	1	0	1	0	1	1	1	0	1	0	1	0	1	0	1	0	0	1
OPI-14	7	2	0	2	0	2	0	2	0	2	0	2	2	5	1	1	3	2	1	2	0
OPJ-19	8	0	0	0	2	0	0	0	2	0	0	0	2	0	0	0	2	1	0	0	3
OPQ-06	8	2	0	0	4	2	0	1	4	2	0	1	5	2	0	0	5	2	0	0	5
Total bands	72	0	1	17	14	9	1	20	16	11	3	18	21	13	3	14	25	11	11	17	18
a + b		11				10				14				16				22			
a + b + c + d		42				46				53				55				57			

No. of primers	Cd concentration (µM)																				
	0	300				450				600				750				1050			
		a	b	c	d	a	b	c	d	a	b	c	d	a	b	c	d	a	b	c	d
OPE-07	8	0	0	0	1	0	0	1	1	0	0	3	1	0	0	1	4	0	0	2	3
OPF-05	9	0	0	0	1	0	0	1	0	1	0	1	3	0	2	5	0	0	2	3	1
OPJ-01	6	2	0	0	4	2	0	0	4	2	0	0	4	2	0	0	6	2	1	2	2
OPJ-20	8	0	0	3	0	0	0	3	0	0	0	3	0	0	0	5	0	0	0	5	0
OPP-06	5	1	0	0	0	1	0	0	1	1	0	0	4	3	0	0	4	1	0	0	4
OPU-20	12	0	0	1	0	0	0	1	0	0	0	0	2	0	0	0	1	0	1	1	0
OPC-08	3	2	0	1	1	0	0	1	1	0	0	1	1	0	0	1	1	0	0	1	1
OPI-14	7	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
OPJ-19	8	0	0	5	0	0	0	5	0	0	0	4	0	0	0	3	0	0	0	0	2
OPQ-06	4	5	0	0	7	5	0	0	7	5	0	0	7	5	0	0	6	5	0	0	6
Total bands	70	10	0	10	14	8	0	14	14	9	0	14	22	10	2	17	22	8	4	16	19
a + b		10				8				9				12				12			
a + b + c + d		34				36				45				51				47			

* a: indicates appearance of new bands, b: disappearance of normal bands, c: decrease in band intensities, and d:increase in band intensities. a+b denotes polymorphic bands, and a+b+c+d, varied band

Almost all of the primers detected more than such alteration in a given sample.

For instance, extra bands of amplified DNA appeared in the case of 300, 450, 600, 750 and 1050 µM cadmium-treated cumin with 5 and 8 primers in Isfahan and Khorasan ecotypes respectively. Five primer namely OPE-05 (one and seven new bands in Isfahan and Khorasan ecotype respectively), primer OPJ-01 (ten new bands in both ecotypes), primer OPP-06 (seven and three new bands in Isfahan and Khorasan ecotype respectively), primer OPC-08 (two and five new bands in Isfahan and Khorasan ecotype respectively) and primer OPQ-06 (twenty five and ten new bands in Isfahan and Khorasan ecotype respectively) produced new band in both ecotypes (Table 2). Primers OPE-07 and OPJ-20 were not amplified any new band under different concentrations of cadmium in two ecotypes. The remanding primers including OPU-20, OPI-14 and OPJ-19 produced five, thirteen and one new bands respectively only in Khorasan ecotype. Extra bands of molecular size from approximately 500–3000 bp were indicated to appear. The number of disappearing RAPD bands increased with the increasing Cd concentration for three primers in Isfahan ecotype and five primers in Khorasan ecotype, and bands of molecular size about 525–2516 bp was shown to disappear. The number of disappeared bands in Khorasan ecotype was much higher than another ecotype. Overall, the number of disappeared RAPD bands correlated positively with the concentration of the cadmium (Table 2).
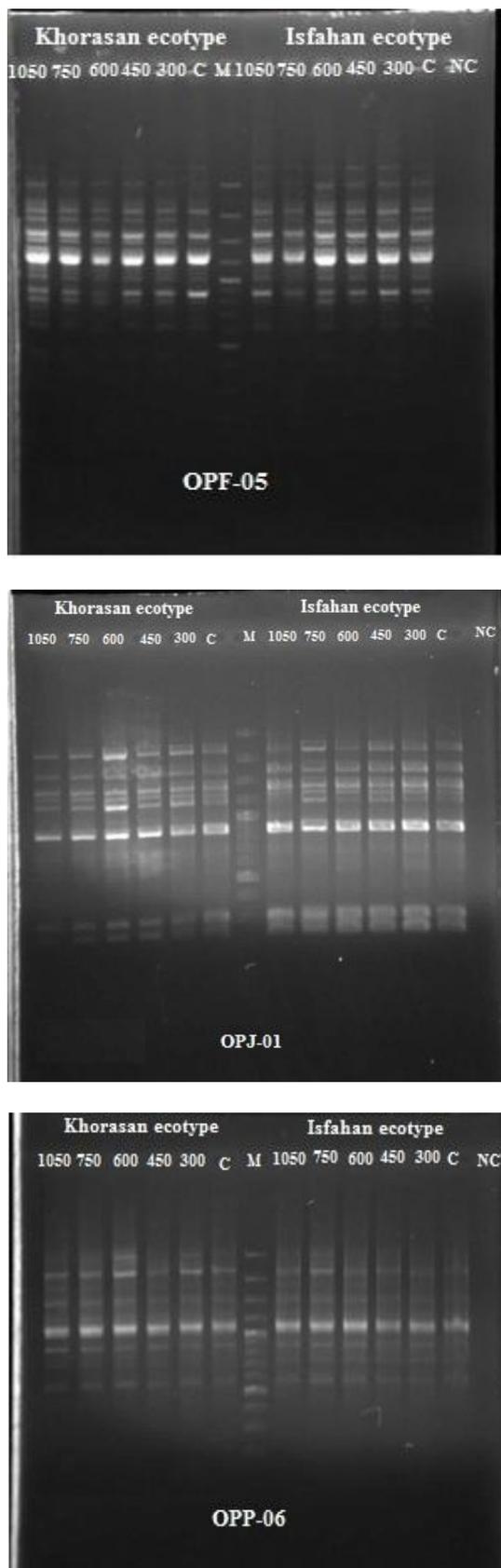
Figure 2. RAPD profiles of genomic DNA from cumin seedlings exposed to varying Cd concentrations (300-1050 μM). NC: negative control, C: control, and M: 100 bp DNA ladder.

Furthermore the decrease and increase in band intensity were particularly obvious for both cumin ecotypes exposed to all cadmium concentration used in this experiment for almost all primers (Table 2).

Ten primers gave a total of 142 RAPD fragments (70 and 72 bands in Isfahan and Khorasan ecotype respectively) ranging from 200-3000 bp in molecular size in the control (Figure 2). Different polymorphic bands were detected at each concentration of Cd for different primers. Value of polymorphisms in Isfahan ecotypes was P (%) = 14.3%, 11.4%, 12.9%, 17.1% and 17.1% for 300, 450, 600, 750 and 1050 μM Cd, respectively. In Khorasan ecotypes the value of polymorphisms of mentioning concentration was 15.3%, 13.9%, 19.4%, 22.2% and 30.5%. The value of observed polymorphism in RAPD patterns in response to Cd stress in Khorasan ecotype was greater than Isfahan ecotype. In all cases, polymorphisms were due to the loss and/or gain of amplified bands in the treated samples compared with the control. Table 2 also indicates that changed bands observed in RAPD profiles (e.g. disappearance, appearance of bands, decrease and increase in band intensity in comparison to control) in the Cd-contaminated cumin seedlings increased dramatically after exposure to Cd. In addition, further experiments confirmed that the variation in band intensities in Figure 2 was stable and not a consequence of either a change in concentration of template DNA within a certain range or a change in PCR reagent concentration (results not shown).

Effect of cadmium pollution on Band Sharing Index (BSI)

The value of Band Sharing Index (BSI) for each treatment group compared with the control was calculated in both ecotypes (Table 3).

Table 3. Effect of Cd on Band Sharing Index (BSA) of two ecotypes compared with control seedlings

Cd concentration (μM)	Isfahan ecotype	Khorasan ecotype
300	0.912	0.889
450	0.937	0.898
600	0.927	0.871
750	0.912	0.869
1050	0.912	0.801

Results showed that in both ecotypes with the increase of Cd concentration following 7 days of treatment, the BSI value was decreased (With the exception of 450 μM concentration). These results reflect the existence of diversity among of RAPD patterns of samples treated with different

concentrations of Cd compared with control. However, the variation in RAPD pattern in response to different concentrations of cadmium was greater in Khorasan ecotype compared with another ecotype. In other words, there was a higher degree of diversity in Khorasan ecotype in response to Cd pollution. However, the band sharing index, despite being very simple to estimate, is not as valuable as GTS index, because it does not take into consideration the absence of bands in profiles.

Effect of cadmium pollution on genomic template stability (GTS) content

Changes in the RAPD patterns were expressed as decreases in GTS, a qualitative measure reflecting obvious changes to the number of RAPD profile generated by the Cd-exposed cumin seedlings, in relation to profiles obtained from control seedlings. A comparison between GTS content of two ecotypes in each treatment is shown in Figure 3. Results indicate that the general tendency of above mentioned index was a progressive reduction with ascending Cd concentration in the culture media. The decreasing trend of the GTS index in Khorasan ecotype was much greater than other ecotype. However the GTS of cumin seedlings increased at 450 μM Cd in both ecotypes only. This effect is ascribed to the multiple changes in RAPD profiles (loss or addition of bands) which tend to counterbalance each other.
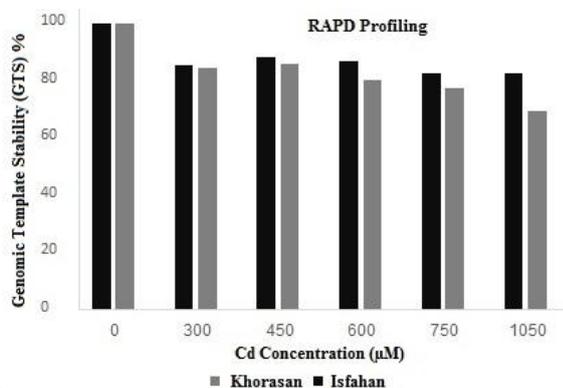
Figure 3. Comparison of genomic DNA template stability in two ecotypes of cumin seedlings exposed to different Cd concentration.

Discussion

Plant growth and development under stress conditions are generally negatively affected. One of these stress conditions that affect plants is heavy metals. Recently, heavy metals have become a hot topic of research for many researchers around the world, mostly due to their detrimental effects on many organisms including plants. Much research has been conducted on the effect of Cd on crops

and other agricultural plants. However, little information is available on the toxicity of Cd on medicinal plants. In ecotoxicology, the effective evaluation and proper environmental monitoring of potentially genotoxic contaminant will be improved with the development of sensitive and selective methods to detect toxicant-induced alterations in the genomes of a wide range of biota. In the present study, the RAPD-PCR technique was used to determine the potential genotoxic effect of cadmium in *C. cyminum*

The results of this study showed that Cd levels affected DNA profile in both ecotypes of *C. cyminum* by different magnitudes and significant changes in RAPD profiles of both ecotypes was observed here.

Once optimized, the use of RAPD for the detection of DNA damage presents a number of advantages. The assay, which is suitable for any extracted DNA of sufficient quality, allows rapid analysis of a large number of samples. As arbitrary primers are used, specific details of DNA damage or the genome sequence in organisms are not needed. Furthermore, no radioactivity or enzymatic degradation of PCR products is required prior to analysis (Atienzar et al., 1999).

The modifications to the genomic DNA were detected by RAPD profiles through the use of randomly primed PCR reactions. These effects include changes in oligonucleotide priming sites and variations in the activity of the *Taq* DNA polymerase. Such effects lead to visible changes in the electrophoretic profiles of RAPD reaction products. The changes in amplified band fluorescence intensity, obvious disappearance of amplified bands and appearance of new PCR products occurred in RAPD profiles generated from the Cd-exposed organisms in comparison to the control (Figure 2 and Table 2)

To test the reproducibility of the RAPD-PCR, the experiments were repeated at least twice for each primer and faint bands were ignored; only reproducible bands obtained in repeated experiments were taken into account.

It has been reported that cadmium can induce a range of DNA damage such a single- and double-strand breaks, abasic sites, modified bases, DNA-protein cross-links, oxidized bases, 8-hydroxyguanine and even bulky adducts representing intra strand dimerization of adjacent purine bases (dimmers) in organisms (Hsiao and Stapleton, 2004; Liu et al., 2007). Dimmers can alter the structure of the DNA (Liu et al., 2007). These DNA damage and structure variations can be better identified by our multiple biomarker

approach presented here.

Modifications of band intensity and lost bands are likely to be due to one or a combination of the following events: (1) changes in oligonucleotide priming sites due mainly to genomic rearrangements and less likely to point mutations and DNA damage in the primer binding sites (because the binding site is only 10 base long whereas genomic rearrangements occur in much longer fragments, e.g. several kb), and (2) interactions of DNA polymerase in cumin seedlings with damaged DNA. These events could act to block or reduce (bypass event) polymerization of DNA in the PCR reaction. The by-pass event is a complicated process that depends on the enzymatic properties of the DNA polymerase, the structure of the lesion and the sequence context of its location (Enan, 2006).

In this experiment, increase in band intensity occurred in the all Cd concentrations for primer OPJ-01, OPP-05, OPJ-19 and OPQ-06 in Khorasan ecotype and for primer OPE-07, OPJ-01, OPC-08 and OPQ-06 in Isfahan ecotype (Table 2). Decrease in band intensity occurred in all used Cd concentrations in this experiment for primer OPJ-20 and OPC-08 in Isfahan ecotype and for primer OPE-07, OPJ-01, OPJ-20, OPU-20 and OPI-14 in Khorasan ecotype (Table 2).

In this study the frequency of band loss was shown to enhance with ascending Cd doses (Table 2).The disappearance of PCR products in some cases affected the high molecular weight bands (i.e. greater than 1 kb) for primers OPE-07 and OPU-20 in Khorasan ecotype and for primer OPJ-01 in Isfahan ecotype because the odds of obtaining DNA damage increased with the length of the amplified fragment. However, some smaller amplicons in this experiment were even much more affected in both ecotypes, thus suggesting a nonrandom interaction between DNA and Cd contamination in soil.

Highest number of disappeared bands that was observed in both ecotypes with cadmium at high concentration (750 and especially 1050 μM) suggests that cadmium maybe cause the same above mentioned changes to the DNA of the treated plants that consequently resulted in the disappearance of DNA bands.

Appearance of new PCR products or appearance of bands could be attributed to the presence of oligonucleotide priming sites which become accessible to oligonucleotide primers after structural change or because some changes in DNA sequence have occurred due to mutations (resulting in new annealing events) or large deletions (bringing two pre-existing annealing sites closer) or

homologous recombination (Atienzar et al., 1999; Enan, 2006). New amplified fragments of mainly greater than 1 Kb in molecular size appeared for all Cd concentrations in both ecotypes. The presence of some extra band in this experiment may also be the results of genomic template instability related to the level of DNA damage, the efficiency of DNA repair and replication (Atienzar et al., 1999).

Our results (Figure 2 and Table 2) indicating increased polymorphism with increasing concentration of Cd, agree well with the observations of other researchers who have also demonstrated a dose-dependent relationship between the DNA changes in treating seedlings and the cadmium concentrations with the RAPD analyses (Liu et al., 2005; Liu et al., 2007; Liu et al., 2009a; Liu et al., 2012). On the other hand, the genomic template stability in the cumin decreased with increasing of Cd concentrations, which indicated that the genomic template stability was significantly affected by Cd stress (Figure 3). Previous studies have also shown that changes in RAPD profiles induced by pollutants can be regarded as changes in genomic DNA template stability and this genotoxic effect can be directly compared with alterations in other parameters (Atienzar et al., 2000). Similar effect on DNA damage was reported due to UV radiation in a marine alga *Palmaria palmate* (Atienzar et al., 2000). Meanwhile, other studies have reported that RAPD or amplified fragment length polymorphism was more sensitive than classic tests such as the comet and micronucleus assay since RAPD analysis was capable of detecting temporary DNA changes at lower concentration of pollutants that may not finally manifest themselves as mutations (Liu et al., 2005; Atienzar and Jha, 2006).

Recently, RAPD technique has been successfully utilized to detect various types of DNA damage and mutation in animals, bacteria and plants induced by low doses of pollutants (Atienzar et al., 1999; Theodorakis, 2001; Atienzar et al., 2002; Rong and Yin, 2004; Liu et al., 2005; Liu et al., 2007; Liu et al., 2009a).

The main advantages of the RAPD method were already mentioned. However, RAPD is only a qualitative method. Effect of each category of DNA damage (e.g. strand breakage, modified bases, abasic sites, oxidized bases, and bulky adducts) on RAPD profiles can only be speculated unless amplicons are analyzed (e.g. sequencing) and more specific methods such as the comet assay and ^{32}P-postlabelling assay is needed to obtain quantitative data (Liu et al., 2009a).

In conclusion the RAPD method has successfully

been used as a sensitive means of detecting Cd-induced DNA damage and showed potential as a reliable assay for soil genotoxicity. From RAPD, ten 10 bp RAPD primers were found to produce a total of 142 RAPD fragments (70 and 72 bands in Isfahan and Khorasan ecotype respectively) ranging from 200-3000 bp in molecular size in the control. The change occurring in RAPD profiles of the seedlings following Cd treatment presents alterations in band intensity, gain or loss of bands compared with control. New amplified fragments of 500–3000 bp in molecular size appeared for all Cd concentrations. The number of missing bands enhanced with the increasing Cd concentration and the genomic template stability reflecting changes in RAPD profiles were significantly affected. This study suggests that the RAPD analysis can be a powerful tool for detection of genotoxic effects of metal contamination in soils.

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Open Access Statement:

Acipenser persicus Growth Hormone Gene Sequencing and its Structures

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Abstract

Administering growth hormone increases growth rate of cultured fish. The aim of this study was isolation and synthesis of Persian sturgeon (GH) cDNA. The total RNA was extracted from pituitary gland of Persian sturgeon and cDNA was synthesized. The full-length cDNA sequence of Persian sturgeon contains a 645 nucleotide open reading frame, which encoding a 214 amino acid protein. The position of the signal peptide cleavage site was predicted to be at position 72. After cleaving of a signal peptide of 24 amino acid residues, a mature peptide of 190 aa formed. The blast observed that the Persian sturgeon pre GH have highest nucleotide sequence similarity with Acipenseridae family and mammals GH. Secondary structure and tertiary structure of Persian sturgeon growth hormone gene were prediction by online software. The secondary structure of the GH revealed the predomination of *a*- helix (> 55%), the domains of high conservation across the vertebrate GH protein. The predicted 3D structure of Persian sturgeon growth hormone attributes to the typical 4-*a*-helix bundle protein conformation, the characteristic 3-D confirmation of growth hormones.

Introduction

Acipenser persicus is an economically important species for high quality meat and caviar production in the Caspian Sea. It belongs to Acipenseridae family and mainly observed in the Iranian rivers, Sefidrood and Gorgan-chaii, flowing into the Caspian Sea (Khoshkholgh et al., 2011). During the late 20th century Persian sturgeon (PS) population rapidly declined and now it's one of the endangered species of the sturgeon fishes so it is under governmental programming using artificial methods to save the species from extinction (Yousefian, 2011). The maturation of this specie is very long however, males reach maturity at 12 - 14 years and females at 14 - 18 years of age. Due to long and costly rearing of growing conditions such as feeding, control of water temperature and dissolved oxygen reduced sturgeon maturity to 7-9 years in males and 9-12 years in females (Huai Tsai, 1995). For acceleration of fish growth and maturation widely used biotechnological approaches such as optimization of culture condition. The Growth hormone (GH) as a potential growth promoting agent has long been recognized and GH administration has been shown to accelerate growth rate in a number of animals, especially fish such as rainbow trout, Atlantic salmon, Nile tilapia, Coho salmon, among others (Huai Tsai, 1995; Luis FM, 2003; Venugopal, 2002). The growth hormone good conserved among animals and fishes but observed high diversity in molecular weights and amino acid sequences and space specify. Hence, the cloning, characterization and expression of GH genes has been the subject of extensive research during the last decade. However, almost all the described GH sequences are for fishes of Europe and western countries (Venugopal, 2002). In contrast, there is a little information on Acipenseridae family growth hormones amino acid sequence and there are no information about the GH genes or amino acid sequence of *Acipenser persicus*. The aim of this study was the synthesis and sequencing GH cDNA of *Acipenser persicus*.

Materials and Methods

In this study four Persian sturgeons were captured in the southern part of the Caspian Sea and transported to the International sturgeon Research

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institute (Rasht, Iran). They were killed and immediately manually extracted their pituitary glands and were used for total RNA extraction.

Total RNA was extracted from pituitary glands of all sturgeons using Biozol solution (Bioflux, Japan) and precipitated into 0.5 volum of RNA precipitation solution (1.2 M sodium chloride, 0.8 M disodium citrate and 0.5 vol of isopropyl alcohol. RNA quality was confirmed using a Nanodrop spectrophotometer with absorbance ratios at OD 260/280. The RNA was treated by DNase and reverse transcribed to first strand cDNA using M-MuLV Reverse Transcriptase kit (Fermentas, USA) and oligo dT(18) at 42 °C for 1 h. All solutions were prepared from DEPC treated autoclaved distilled water. The PS preGH cDNA of all fishes were sequenced in Bioneer Co., South Korea and no differences between the male and female's pre GH cDNA sequences were found.

The specific primers used for amplification of cDNA encoding the target genes were designed from alignment of several known acipenseridea GH nucleotide sequences and matches with the first exon region which was retrieved from the NCBI GenBank. [*Acipenser sinensis* (EU119864.1), *Acipenser gueldenstaedtii* (Russian Sturgeon) (AY941176.1) and *Huso Huso* (AB517597.1). The primers were 5'-ATGGCATCAGGTCTGCTT (forward primer) and 5'-CTACAGAGTACAGTTGCTCTC (reverse primer).

The PCR reaction was Primer sets were generated using Primer3 program (http://biotools.umassmed.edu/bioapps/primer3_www.cgi). The PCR was performed under the condition of DNA denaturation at 94°C (5 min), followed by 35 cycles of denaturation at 94°C (30 sec), annealing at 58°C (90 sec) and extension at 72°C (30 sec), with a final extension at 72°C (25 min). Amplicons were separated by 1.5 % agarose gel electrophoresis and stained with Ethidium bromide.

PCR products of the appropriate size was excised from the gel, purified by an extraction kit (Vivantis, Iran) and sent to Takapozist Company(Iran) for sequencing.

Nucleotide and deduced amino acid sequences were analyzed using BLAST-N and BLAST-P (GenBank, NCBI, http://www.ncbi.nlm.nih.gov). The signal peptide and putative cleavage sites were detected using the Signal-P (http://www.cbs.dtu.dk/services/SignalP). N glycosylation sites were prognosticated by searching the Asp-Xaa-Ser/Thr motif (http://www.cbs.dtu.dk/services/ NetNGlyc). Secondary structure and tertiary structure of PS GH

gene were predicted by online software (Guermeur, 1999; Ma J, 2013).

Results

In our study we isolated and synthesized GH cDNA of Persian Sturgeon. The Persian sturgeon preGH cDNA contains an open reading frame of 645 nucleotides starting from ATG codon and ending with TAG stop codon encoding preGH of 214 amino acid residues. The first 24 amino acid residues from the N-terminus are highly hydrophobic (~70% of the amino acids residues of this region are non-polar) and also have a high degree of homology to the signal peptide of other fish GHs, it is assumed that in the Persian sturgeon pre-GH this region probably represent the signal peptide which is cleaved upon hormone secretion. The position of the signal peptide cleavage site was predicted to be at position between 24 and 25 amino acids. After cleaving of the signal peptide formed mature GH containing 190 amino acid residues starting with a glutamic acid (figure 1).

The obtained polypeptide exhibit typical GH feature, such as four Cystein residues, capable of forming two disulfide bonds which are assumed to contribute to the tertiary structure of the hormone, a single tryptophan residue and stretches of amino acid highly conserved in all known GHs. There is only one Asn-Xaa-Thr amino acid motif in GH at the C terminus region which is potential site for N-linked glycosylation. The amino acids Leucine and Serine are dominant, about 25 % of total pool and only one tryptophan is exists.

By means of Sequences Producing Significant Alignments from National Center for Biotechnology Information (NBCI) data base we compared the sequences of PS GH gene with other fish as well as mammalian GH genes sequences, which demonstrated high degree of identity, especially with Acipenseridea's GH sequences, scores denote conserved nucleotides (71–99%) (Table 1).Thus, the GH coding DNA sequences of Persian sturgeon GH have 99% similarity to Beluga cDNA (*Huso Huso*) GH, 72% to *Sus scrofa* and 73% to mouse. Apart of a few deletions and insertions, GH is a remarkably conserved protein. The molecule is composed of four conserved region and four variable regions which are likely to be functionally important. In contrast to Persian sturgeon and other fish species the GH of goldfish and in other Cyprinidae contain 5 Cystein residues. Since the gene GH is a highly conserved protein, it provided a better resolution for more distantly related animals. The characteristics some of vertebrates are presented in table 2.

Atg	gca	tca	ggt	ctg	ctt	ctg	tgt	cca	gtg	ctg	ctg	gtt	ata	ttg	ctg	gtc	tcc	cct	aaa
M	A	S	G	L	L	L	C	P	V	L	L	V	I	L	L	V	S	P	K
Gag	tct	ggg	gcc	tac	cct	atg	att	cca	cta	tcc	agt	ctt	ttc	aca	aac	gct	gtg	ctc	aga
E	S	G	A	>Y	P	M	I	P	L	S	S	L	F	T	N	A	V	L	R
Gca	cag	tac	cta	cac	cag	ctt	gct	gca	gac	att	tac	aaa	gat	ttc	gag	cgt	acc	tat	Gtt
A	Q	Y	L	H	Q	L	A	A	D	I	Y	K	D	F	E	R	T	Y	V
cca	gat	gag	caa	cgt	cac	tcc	agc	aaa	aac	tcc	ccg	tca	gca	ttc	tgc	tac	tct	gag	acc
P	D	E	Q	R	H	S	S	K	N	S	P	S	A	F	C*	Y	S	E	T
atc	cct	gct	ccc	act	ggc	aaa	gat	gag	gcc	caa	cag	cga	tca	gac	gtg	gag	ctg	ctt	cag
I	P	A	P	T	G	K	D	E	A	Q	Q	R	S	D	V	E	L	L	Q
ttt	tcc	ctg	gct	ctc	atc	cag	tcc	tgg	att	agt	ccc	ctg	cag	tcc	ctg	agc	cgt	gtt	ttc
F	S	L	A	L	I	Q	S	W	I	S	P	L	Q	S	L	S	R	V	F
acc	aat	agc	ctg	gtg	ttc	agc	acc	tcc	gac	cga	gtg	ttt	gag	aaa	ctg	aaa	gat	ctg	gag
T	N	S	L	V	F	S	T	S	D	R	V	F	E	K	L	K	D	L	E
gaa	ggc	att	gtg	gct	ctc	atg	agg	gat	ctg	ggg	gaa	ggc	ggt	ttc	gga	agt	tct	act	ttg
E	G	I	V	A	L	M	R	D	L	G	E	G	G	F	G	S	S	T	L
ctg	aag	ctc	act	tat	gat	aag	ttt	gat	gtc	aac	cta	aga	aac	gat	gat	gct	ttg	ttt	aaa
L	K	L	T	Y	D	K	F	D	V	N	L	R	N	D	D	A	L	F	K
aat	tat	ggg	ctt	tta	tgc	tgt	ttt	aag	aaa	gat	atg	cac	aaa	gta	gag	acg	tac	ctg	aaa
N	Y	G	L	L	C*	S	F	K	K	D	M	H	K	V	E	T	Y	L	K
gtg	atg	aaa	tgc	aga	cgt	tgt	gtg	gag	agc	aac	tgt	act	ctg	tag					
V	M	K	R	R	F	C*	V	E	S	N	C*	T	L						

Figure 1. Persian sturgeon (*Acipenser persicus*) preGH ORF complete nucleotide and deduced amino acid sequences. The potential glycosylation site is underlined. The arrow indicated the possible site for cleavage of signal peptide. The cysteine residues in the mature hormone are asterisked

Table 1. Sequences producing significant alignments from (NBCI) data base

Accession	Family	Species	Max identity
JN604534.1	Acipenseridae	*Acipenser persicus*	100%
HQ166628.1		*Huso huso*	99%
AY941176.1		*Acipenser gueldenstaedtii*	98%
KC460212.2		*Acipenser schrenckii*	98%
JX947839.1		*Acipenser baerii*	98%
EU599640.2		*Acipenser sinensis*	96%
EU390781.1	Mammalia	*Sus scrofa*	72%
AF052192.1		*Trichosurus vulpecula*	72%
X02891.1		*Mouse (Mus musculus)*	73%
S50877		*Ovis aries*	71%
V01237		*Rattus norvegicus*	71%
V00520		*Homo sapiens*	74%
EF521480.1	Aves	*Eupodotis ruficrista*	92%
EF521592.1		*Scytalopus magellanicus*	81%
Ay148493	Anguilidea	*Anguilla anguilla*	67%
M24066		*Anguilla japonica*	67%
M24683	Salmonidea	*Onchorhynchus mykiss*	65%
S52027	Amphibia	*Rana catesbiana*	67%
X60475	Cypriniformes	*Carassius auratus*	61%
M27000.1		*Cyprinus carpio*	65%
X60475		*Hypoththalmichtys mulitrix*	68%
AF389237		*Pimephales promelas*	68%

.

Table 2. The GH characteristics some of vertebrates

Character	PS	Huso huso	Russian
Nucleotides	967	967	980
5' UTR	25	25	39
ORF	645	645	642
Stop codon	TAG	TAG	TAG
Protein	214	214	213
Signal peptid	25 aa	25 aa	24 aa
Mature protoein	190 aa	190 aa	190 aa
Mol. mature Wt (kDa)	~22 kDa	~22 kDa	~ 22kDa
Isoelectric point (pH)	6.31	6.31	6.31
Glycosylation sites	187	187	187
Cystein residues	76, 187, 204 , 212	76, 187, 204 , 212	76, 187, 204 , 212

In order to make the PS preGH phylogenetic tree with the GHs of other species, at first PS preGH and several known GHs genes of many species from different species in NBCI were blasted together. The blast revealed that the PS preGH have highest nucleotide sequence similarity to other GH sequences. Thus, Acipenseridae family (98-99%), mammals GH (72%) and Brides GH (*Gallus gallus*) (73%) as well as by Muse (73%), *Trichosurus vulpecula* (72%) (Table 1).

The secondary structure of the GH was determined by in homogenous score combination method of Guermeur *et al.* (1999) based of neural networks (http://npsa-pbil. ibcp.fr/npsa) and by the PROFILESCAN, PEPTIDESORT and other modules of the GCG software (Figure 2).

It is revealed the predomination of *a*- helix (> 55%), the domains of high conservation across the vertebrate GH protein, which attributes to the characteristic 4-*a*-helix bundle confirmation. The four different key *a*-helices are indicated by roman numerals. Second predominant random coils (35%) which connect the helices support the 4 *a*-helix bundle confirmation.

The predicted 3D structure of psGH was made by template-based modeling including alignment and template selection (Ma J, 2013) availeble (http://raptorx.uchicago.edu/download/.) (Figure. 3).

The 56.07% of amino acids are involved in *a*-helix formation, which run anti-parallel to each other. These helices attribute to the typical 4-*a*-helix bundle protein conformation, the characteristic 3-D confirmation of growth hormones.

Figure 2. The predicted secondary structure of the PS GH protein. Protein sequence is annotated with secondary structure information [a-helix in blue, (H); extended strand in red, (E); random coil, (C)].
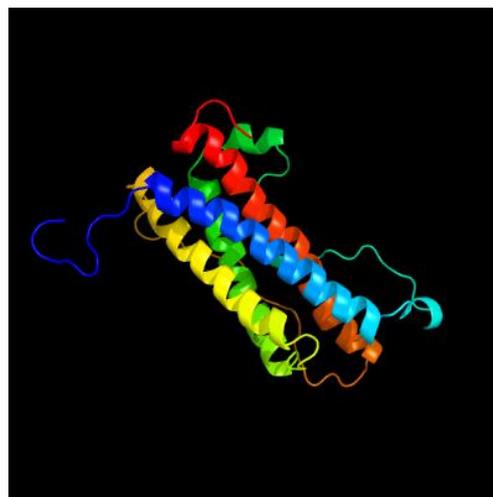
Figure 3. 3-D structure of the mature PS GH.

Discussion

In this study we described sequence analysis and of Persian Sturgeon GH to other vertebrates. Apart from a few deletions and insertions, GH is a remarkably conserved protein. GH molecule is composed of four conserved and four variable regions which are likely to be functionally

important. Since the GH gene is a highly conserved protein, it provided a better resolution for more distantly related species (Luis FM, 2003; Venugopal, 2002).

As a result, the aa sequences of Persian sturgeon (PS) GH have 99% similarity to beluga (Huso Huso) and highest (99%) levels of homology to the GHs of Acipenseridea and mammalian.

The comparison of aa amounts shows no difference between GH of PS and RS and only a little difference between PS and Huso huso. The cystein residues, which are important for the disulfide bond formation and structural integrity of the 3-D structure of the GH protein (Schneider, 1992) is conserved in sturgeons and located at 56, 146,187 and 192 positions. Their presence was found to be important for the structural integrity and biological activity of the hormone. Probably these are the regions, from which strong homology could be drawn between vertebrate GH sequences (Schneider, 1992).

Primary Structure of PS GH Gene contains an ORF nucleotide sequence of the gene (645 bp) and matur sequence (570 bp) with 190 amino acid was determined. The position of signal peptide was in 72 nucleotide (24 amino acid) (fig.1) and this sequence registered in gene bank NCBI for the first time and was given number JN604534.

In the Russian sturgeon (*A. gueldenstaedtii*) growth hormone cDNA nucleotide sequence was 980 bp long and had an open reading frame of 642 bp, beginning with the first ATG codon at position 39 and ending with the stop codon at position 683. The position of the signal peptide cleavage site was predicted to be at position 111, yielding a signal peptide of 24 amino-acids (aa) and a mature peptide of 190 aa. (Yom Din, 2008).

The Beluga sturgeon (*Huso huso*) growth hormone cDNA also has an open reading frame of 645 nucleotides encoding a protein 214 amino acid residues. The signal peptide cleavage site was predicted to be at position 72, yielding a signal peptide of 24 amino acid residues and a mature peptide of 190 amino acids. The cDNA sequence of the Russian sturgeon was similar to that of the Beluga cGH. (Azizzadeh, 2013).

Cao H. et al. (2011) show that the Chinese sturgeon *A. sinensis* GH cDNA consists approximately 954 bp in size including a 16 bp 5′-untranslated region and 296 bp 3′-untranslated region. The open reading frame (642 bp) encodes a 214 aa, but it represents the precursor composed of a 25 aa signal peptide followed by a 189 aa mature polypeptide (Cao, 2011).

Conclusion

These results provide useful information for P.S GH, the finding demonsterate that the GH sequence have a higher degree of identity to mammalian and other fish. These are the first time an *Acipenser persicus* growth hormone encoding cDNA (PSGH; GenBank no. JN604534.1) has been reported and the deduced amino acid sequence obtained. Keeping in view the present results we are here able to suggest future experimental focuses.

Acknowledgment

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Open Access Statement:

Evaluation of Agrobacterium-mediated Transformation of *Chlamydomonas reinhardtii* Using a Synthetic Amorpha-4, 11-diene Synthase Gene

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Abstract

Amorpha-4,11-diene synthase (ADS) is a key enzyme in biochemical pathway of the antimalarial agent artemisinin. An Agrobacterium-mediated transformation was carried out to express a synthetic *ADS* gene in green microalga *Chlamydomonas reinhardtii* strain 125C with bacterial strains GV3101 and LBA4404. The foreign gene was optimized based on codon usage bias of the microalga. Integration of the *ADS* in nuclear genome of *C. reinhardtii* was confirmed by polymerase chain reaction assay. The transgenic colonies cultured on selective medium turned yellow after three days and gradually died. Transformation procedure, growth habit of the transgenic microalgae together with probable causes of transformants loss is discussed. The present study is the first investigation for production of ADS enzyme in a microalgal system.

Introduction

In many regions of the world especially in developing countries malaria is a prevalent infectious disease which is the tenth cause of death and is predicted to remain at that level until at least 2030 (Castello et al., 2010). It is estimated that more than 380 million cases of malaria occur annually accounting for more than 1 million deaths (Briolant et al., 2010).

The most potent treatment for malaria disease is an herbal compound called artemisinin and its derivatives. Artemisinin is a secondary metabolite produced in the herbaceous plant *Artemisia annua*. Artemisinin has a short half-life so significant quantities in prescribed doses is required and thus there is an increasing demand for this important therapeutic agent (Crockett et al., 2007). Moreover, although artemisinin is still the preferred treatment for high risk patients, its use is hampered by its high cost, because is produced a very low amount of this compound in *A. annua*. Enhancing artemisinin concentration in plant tissues is,

therefore, a practical way to make artemisinin available as a cheap antimalaria drug (Wallaart et al., 2000). An ideal approach to achieve this goal would be genetic engineering of enzymes involved in biosynthesis of artemisinin. Artemisinin is synthesized in plant tissues via a complex pathway including different enzymes, the most critical of which is amorpha-4,11-diene synthase (ADS) which catalyzes conversion of farnesyl diphosphate (FPP) in to amorpha-4,11-diene (Arsenault et al., 2008).

Considering low content of artemisinin in *A. annua* tissues and high cost of synthetic production of the product on one hand, and increasing demand for artemisinin on the other hand; there have been extensive investigations for expression of artemisinin complex precursors such as Amorpha-4,11-diene in microbial platforms such as *E. coli* and *Saccharomyces cerevisiae* and even in the higher plant tobacco (*Nicotiana tabacum*) aiming at achieving a cost-effective way for production of artemisinin (Khosla et al., 2003).

Although production of artemisinin precursors in bacterial and yeast hosts has been successful, large scale production of artemisinin in such systems which requires vitamin and carbon source is not cost effective (Walker et al., 2005). This limitation can be circumvented by adopting an appropriate

host such as fresh water microalgae *Chlamydomonas reinhardtii*.

Application of microalgae for production of pharmaceuticals offers a number of advantages not found in other production systems. Microalgae are cost effective platforms for therapeutics production and are free of human or animal pathogens (Dove, 2002; Tran et al., 2009). Moreover, they possess elaborated cellular machinery required to fold complex human proteins that bacteria and yeast may not be able to process properly (Rosenberg et al., 2004). Many species of green algae are generally regarded as safe (GRAS) microorganisms, meaning that if the protein can be expressed in a bioavailable form, purification steps could potentially be eliminated altogether. This will result in further reduction of production costs (Rasala et al., 2010).

Microalgae can be grown in contained bioreactors, reducing the risk of contamination of the production system by airborne contaminants, and also protecting the environment from any potential flow of transgenes into the surrounding ecosystem. Time course from initial transformation of microalgae to large-scale protein production is only a matter of weeks, compared to months or years in higher plants (Franklin and Mayfield, 2004). Indeed, algal-based platforms combine the advantages of microbial and plant systems for production of therapeutic agent (Rasala et al., 2010).

Genetic transformation of microalgae can be carried out in many ways including microparticle bombardment, glass beads, electroporation and Agrobacterium-mediated transformation (Soria-Guerra et al., 2013). Since the pioneer work of Kumar et al (2004), Agrobacterium-mediated transformation has been widely used for genetic manipulation of microalgae. This method is a simple and yet efficient approach that can be carried out in a relatively short time with low cost (Specht et al., 2010).

The goal of the present study was to investigate production of amorpha-4,11-diene as a major precursor of artemisinin in *C. reinhardtii* using Agrobacterium-mediated genetic transformation.

Materials and Methods

Construct design

Nucleotide sequence of *ADS* gene was obtained from NCBI website (http://www.ncbi.nlm.nih.gov/) with accession number of AF138959.1 and the corresponding protein sequence was obtained with accession number of AAF61439.1. The coding

sequence was optimized using Vector NTI advance 11 software. This sequence encodes for the 63.933 kD ADS protein. To facilitate cloning procedure in pBI121 plasmid, *Bam*HI and *Sac*I restriction sites were included in 5´ and 3´ ends of the gene, respectively. The *ADS* gene was synthesized in pGEM vector (Bioneer, south Korea).

Construction of expression vector

The synthetic *ADS* gene was removed from pGEM vector by digestion with *Bam*HI and *Sac*I and inserted into the plant expression vector pBI121, downstream of the CaMV 35S promoter and upstream of the nopaline synthase (NOS) terminator, yielding pBI121-ADS vector. *Bam*HI/*Sac*I double digestion removes *GUS* gene from pBI121 plasmid.

The resulted vector was used to transform *E. coli* strain DH5-α and kanamycin-resistant colonies were selected after overnight incubation at 37°C. For confirmation of the recombinant plasmid pBI121-ADS, at the first the plasmid was extracted from bacterial cells using alkaline lysis method, then digestion of recombinant plasmid with *Eco*RI and PCR analysis of the recombinant plasmid using gene specific-forward primer and NOS terminator-specific reverse primer was performed. The sequence of forward and reverse primers: 5' TAAATGGGCTAATGAAGGTC 3' and 5' GAAGAAAGCGAAAGGAGC 3', respectively. PCR was carried out as follow: 94 °C 45s, 52 °C 45s, 72 °C 1.5 min, 30 cycles.

pBI121-ADS vector was introduced into *Agrobacterium tumefaciens* strain GV3101 and LBA4404 by thaw-melting method. Transformed cells were selected on screening medium (LB medium containing 50 mg/l kanamycin and 50 mg/l rifampicin) and colony PCR was carried out similar to that already explained.

Chlamydomonas culture

Chlamydomonas strain 125C was obtained from Chlamydomonas Resource Center (University of Minnesota). Algal cells were grown in Tris-Acetate-Phosphate (TAP) medium under continuous cool fluorescent light at 25°C.

Genetic transformation of *C. reinhardtii*

C. reinhardtii cells were transformed using the method proposed by Kumar et al (2004) with some modifications. In summary, 10^7 *Chlamydomonas* cells were transferred on to the solid TAP medium with 100 μM and without acetosyringon (AS) and incubated in light for 2 days to form algal lawn. A fresh *Agrobacterium* culture (A600 = 0.5) grown in

liquid LB medium containing appropriate antibiotics (50 mg/l rifampicin and 50 mg/l kanamycin) was centrifuged in 4500rpm for 10 min, the supernatant was discarded and the bacterial pellet was resuspended in 200 μl TAP broth with or without AS. The bacterial suspension was spread to the thin layer of *Chlamydomonas* lawn growing on agar plate. The co-cultivation plates were incubated for 2 days at 25°C. The cells were then harvested and washed twice with liquid TAP medium containing 500 mg/l cefotaxime to eliminate bacterial residues and centrifugation at 1000 rpm for 2 min, resuspended in liquid TAP. The washed *Chlamdomonas* cells were cultured in solid selection medium containing 50 mg/l Kanamycin and 500 mg/l cefotaxime.

PCR screening of transformants

Half of each transformant colony was screened for presence of the gene of interest using aforementioned primers. *Sac*I digestion was carried out to confirm PCR products. Cells were resuspended in Tris-EDTA solution and heated to 95°C for 10 minutes. The cell lysate was used as a template for PCR reactions. The other halves of the colonies with positive PCR results were grown in liquid selection medium containing 50 mg/l Kanamycin for further analysis.

Results

Design and construction of expression cassette

After codon optimization of *ADS* gene and inclusion of *Bam*HI and *Sac*I restriction sites in 3´ and 5´ ends, the optimized 1653 nucleotide sequence was synthesized and cloned in pGEM vector. After digestion of pGEM-ADS and pBI121 vectors with abovementioned enzymes, the *ADS* gene was inserted in pBI121 vector (figure1).
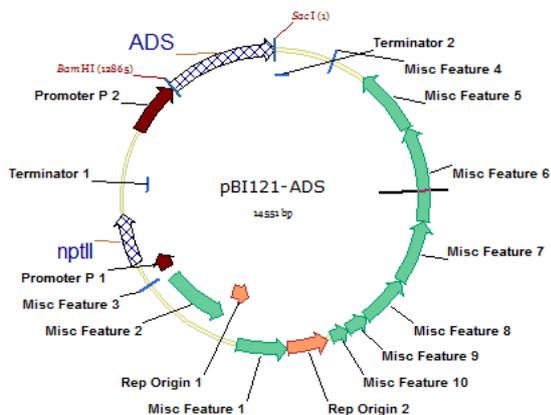
Figure 1. Schematic components of pBI121-ADS expression

vector

After transfer of pBI121-ADS in to *E.coli* cells, the extracted plasmid of the putative transformed cells were evaluated using digestion with *Eco*RI restriction enzyme (figure 2) and PCR with specific primers (figure 3). *Eco*RI endonuclease has two restriction sites on the recombinant plasmid; one on pBI121 plasmid and the other on *ADS* gene.
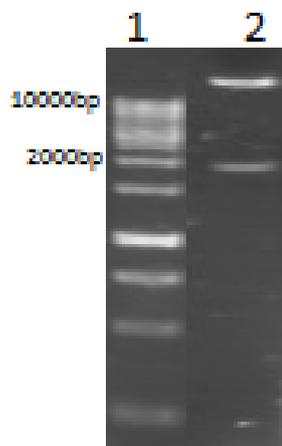
Figure 2. Digestion product of *Eco*RI. lane1: 1kb ladder (Fermentas); lane 2: digestion pBI121-ADS with *Eco*RI (12600bp and 1900 bp segments).
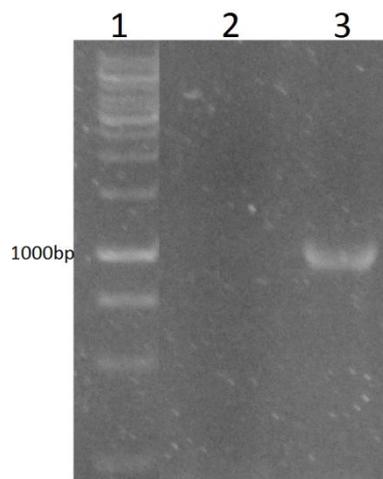
Figure 3. lane1: 1kb ladder (Fermentas); lane 2: negative control (*E.coli*); lane 3: Transformed cell (The 1000bp band resulting from PCR with specific primers)

Genetic transformation of *C. reinhardtii*

Genetic transformation of *C. reinhardtii* was carried out using two strains of *A. tumefaciens* namely LBA4404 and GV3101. The transformed colonies appeared on selective medium after 7-10 days. In the lack of acetosyringone treatment, 2-3 colonies were obtained for both strains of Agrobacterium. However, by application of acetosyringone (100 μM), the number of transformants increased significantly, that is 270 and 350 colonies were achieved for LBA4404- and

GV3101-mediated transformation, respectively.

PCR assay

Polymerase chain reaction (PCR) was performed to evaluate presence of foreign gene in putative transgenic microalgal cells. For each strain of Agrobacterium, twenty colonies were used for colony PCR among which, seven colonies showed the expected band (figure 4). In evaluation of PCR product using digestion with *Sac*I restriction enzyme, desired segments was observed. (figure 5)
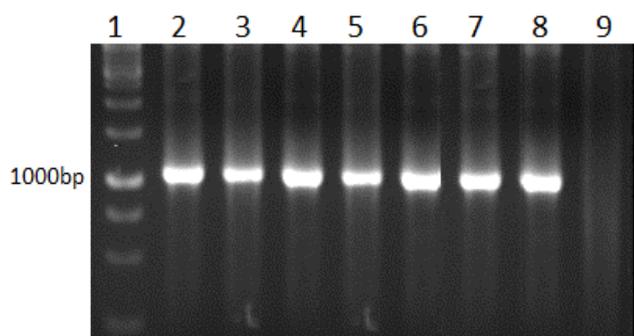

Figure 4. Gel electrophoresis of colony PCR products. Lane 1: 1kb ladder (Fermentas); lanes 2-5: colonies transformed using GV3101; 6-8: colonies transformed using LBA4404 and lane 9: wild type
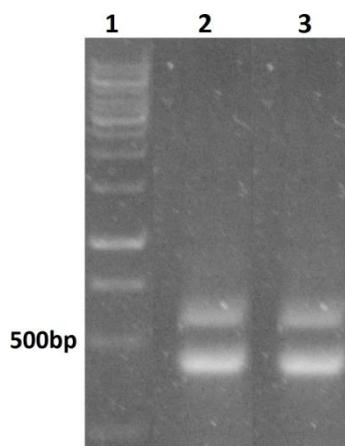
Figure 5. Digestion of PCR product with *Sac*I. lane1: 1kb ladder (Fermentas); lane 2-3: digestion of PCR product with *Sac*I (600bp and 400bp segments).

Growth kinetic of transformant colonies

As stated in Material and methods section, half of each transgenic colony was cultured in selective broth medium. The colonies showed normal growth in the first three days creating a bright green color; however the cells got yellow thereafter and were completely lost on the seventh day. The experiment was repeated for more colonies but the results were the same. Some colonies even showed no growth in selective medium. The lack of growth in selective liquid media prevented from subsequent analyses such as expression assay.

Discussion

In this study, we investigated application of green microalgae *C. reinhardtii* as a platform for expression of ADS as a key enzyme in biochemical pathway of artemisinin. Although heterologous expression of ADS has been reported in other biological systems (Arsenault et al., 2008), to the best of our knowledge the present study is the first investigation for production ADS enzyme in a microalgal system. The synthetic *ADS* gene was optimized based on codon usage of *C. reinhardtii*. It has been reported that low expression of foreign genes in algal system is often due to the incompatibility of the codon usage in their coding regions which consequently decreases the efficiency of the translation (Mayfield et al., 1990). Although we were not able – due to loss of transgenic lines- to analyze the recombinant protein in translation level, some results achieved in this investigation worth noting. Acetosyringone had significant influence on the percentage of transformants, increasing from 2-3 colonies in 0μM to 350 colonies in 100μM of acetosyringone. This is in accordance with the results reported by Kumar et al (2004) who found out that 100μM of acetosyringone significantly enhanced transformation percentage.

Application of GV3101 strain led to better results compared to LBA4404 strain. Similar to our results, in a recent investigation it was reported that GV3101 was effective in increasing transformation rate (Habibi-pirkoohi et al, 2014). However, in another study investigating efficacy of different strains of *A. tumefaciens* in genetic transformation of microalgae, the best results were achieved by strain EHA 105 (Pratheesh et al., 2012). It seems that adoption of a certain strain as the best candidate for transformation of microalgae is not conclusive.

Another feature of the present study was instability of transformed cells of *C. reinhardtii*. The green colonies of transformed cells gradually turned in to yellow about two weeks after transformation procedure. Instability of microalgal transformed colonies has been reported by other authors (Leon et al., 2007; Habibi et al., 2014). Generally, in chlorophytes such as *C. reinhardtii*, transformation frequency of heterologous constructs is very low and gene expression is instable (Neupert et al., 2009). The unique nuclear characteristics of these microalgae can influence their ability to express genes under the control of heterologous promoters

(von Gromoff et al., 2008).

It is generally accepted that in chlorophytes and diatoms, stable expression of heterologous genes can only be optimally achieved when adequate homologous promoters or promoters from very close species are included (Shao and Bock, 2008).

A great number of regulatory elements and transcriptional or post transcriptional events can influence on the expression level of the transgenes and on their stability (Kathiresan et al., 2009). Expression of an exogenous gene can be very low or null, even though all the elements required for optimal transcription and translation -promoters, introns and other regulatory regions- have been included in the chimeric gene construction. Difficulties for foreign gene expression in microalgae can be due to the lack of adequate regulatory sequences, to positional effects, biased codon usage, incorrect polyadenylation, inappropriate nuclear transport, instability of the mRNA, or gene silencing (Rasala et al., 2010).

In the present study, the transformed colonies were cultured on selective media; whereas in the pioneer work conducted by Kumar et al (2004), the transformants were cultured in non-selective medium. It has been reported that when transgenic algal clones are not maintained under selection conditions, expression of the exogenous genes might be suppressed (Leon et al., 2007). Furthermore, to verify reliability of the results reported by Kumar et al (2004), we cultured some PCR-positive clone in non-selective media. The colonies underwent normal growth, but after full growth no band was observed for these colonies in PCR assay, suggesting that the transformants lost the transgene during growth in non-selective medium. The results obtained in the present investigation can be justified by transient expression theory. Transient expression of firefly luciferase (Luc) in protoplasts of *Chlorella ellipsoidea* and of β-glucuronidase (*Gus*) gene in *C. saccharophila* has been already reported (Leon et al., 2007).

It can be postulated that expression of ADS exerts a metabolic load on *C. reinhardtii* cells and this suppresses microalgal growth. However, since the growth of transformants on selective media was normal for a few days, the hypothesis of metabolic load can't be so reliable. Therefore, we believe that instable gene expression is the probable cause of the results observed in the present study.

As a conclusion, agrobacterium-mediated procedure is a simple and cost-effective method for nuclear transformation of microalgae and an acceptable number of colonies is achieved in this way. However, stable expression of ADS- and other foreign genes- in *C. reinhardtii* using agrobacterium-mediated transformation requires more elaborated constructs and algae-specific regulatory elements specially homologous promoters (Spetch et al., 2010).

Although gene expression was not analyzed in this investigation, based on the same results obtained in duplicate experiments and similar number of colonies achieved with two different Agrobacterium strains, it can be concluded that further studies along with application of homologous elements are required to achieve appropriate and stable results in nuclear transformation of *C. reinhardtii* moreover, it appears that combating gene silencing will be a major hurdle before recombinant proteins can be expressed at economically viable levels from nuclear transgenes in *Chlamydomonas*. (Specht et al., 2010).

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Open Access Statement:

Recombinant Vaccine Production in Green Plants: State of Art

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Abstract

Green plants have emerged as ideal platforms for production of recombinant vaccine during recent decades. Various antigens relating to a large number of animal and human diseases have been studied in different plant species for production of recombinant vaccines. Despite the unique advantages of plant systems as green factories for production of recombinant vaccines, there are some major hurdles that have prevented commercial production of plant-based vaccines. In this review, theoretical background and practical applications of plant system for production of various recombinant vaccines are discussed.

Introduction

Plant-derived pharmaceuticals (PDPs) are proteins or organic compounds produced in plants via recombinant DNA technology, which are used to improve human or animal health. Subunit vaccines represent one category of PDPs that have been validated in a variety of studies, including human clinical trials. Application of green plants for production of therapeutic products is an emerging field of biotechnology with high economic potential (Sala et al., 2003). Although vaccination with conventional vaccines proved to be an effective practice in prevention of diseases, yet there still is disagreement over its use. Some of the documented side effects of the elements and substances used in vaccine serums include: blood disorders, auto-immune diseases, cerebral palsy, brain damage, paralysis, neurological impairment, monkey fever, autism, mental retardation, premature aging, as well as others. Thus, there is an urgent need to find an alternative to the present vaccines. This alternative can be substituted by development of plant vaccines (Schillberg et al., 2005).Considering recent developments in genetic engineering and transformation methods, it is possible to develop a wide range of transgenic plants that can express various recombinant pharmaceutical compounds

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including viral and bacterial antigens, antibodies, and many other therapeutic proteins (Awale et al., 2012).

For a long time, Recombinant vaccines were exclusively produced in expensive expression platforms such as yeast or mammalian cells. High costs associated with preparation culture media and the risk of contamination by human pathogens are regarded as the major disadvantages of such systems. Production of recombinant vaccines in bacterial systems, though simple and cost-effective, was not successful due to improper folding of eukaryotic peptides and occurrence of inclusion bodies in bacterial hosts (Franklin and Mayfield, 2005). Genetic engineering of higher plants was a turning point in the field of recombinant vaccine production. The goal is to produce transgenic plants that upon oral or parenteral administration induce an immune response in the body. The first report of expressing a vaccine antigen within plants was published in 1990 when Curtiss and Cardineau expressed the Streptococcus mutants surface protein antigen A (SpaA) in tobacco (Curtiss and Cardineau, 1990). This pioneer study was followed by plant expression of the hepatitis B surface antigen (HbsAg) (kapusta et al., 1999), the *E. coli* heat–labile enterotoxin responsible for diarrhea (Haq et al., 1995), and the rabies virus glycoprotein (McGarvey et al., 1995). Proteins produced in these plants induced synthesis of antigen specific mucosal IgA and serum IgG when delivered orally to mice and humans.

Compared to other recombinant protein expression systems, Plants offer several advantages including the possession of eukaryotic posttranslational modification machinery, suitable folding of foreign protein, low cost scale up, target protein stability and safety of use of plant-derived products due to the lack of any mammalian pathogens. The cost of vaccine production in plant systems is comparable to that of microbial bioreactors and much lower than in mammalian cells. More importantly, in contrast to microorganisms, especially bacteria, it was well documented that plants express eukaryotic proteins in properly folded, modified, assembled and, consequently, native and biologically active forms. Plant-based recombinant vaccines are also advantageous in terms of safety, as naturally free of microbial toxins and human and animal pathogens (Pniewski et al., 2012). However, oral immunization is thought to be the largest benefit and, in the most enthusiastic plans, plant-based vaccines are to be used as edible vaccines(Awale et al., 2012).
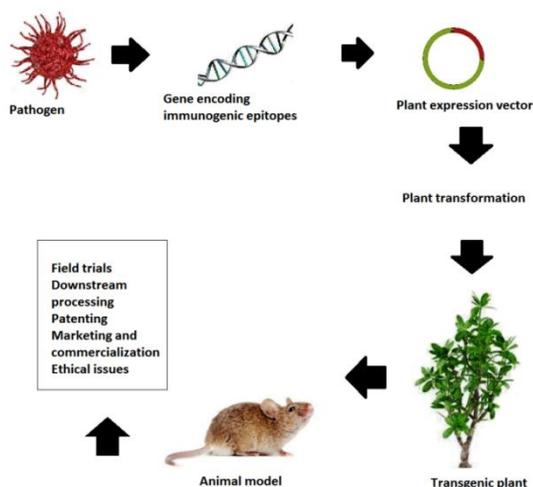
Figure1. Schematic representation of recombinant vaccine production in plat systems

Here, we first describe the principles of plant-based recombinant vaccine production. A handful examples demonstrating successful expression of antigen in plants are also cited. Moreover, strategies toward enhancing expression level will be noted, and finally, biosafety issues and future perspectives for commercial production of recombinant vaccines are discussed.

Plant species used for vaccine production

Plant-based vaccines are subunit vaccines in which the antigen of interest is expressed in plant tissues. The antigen, or antigens, must be expressed at a sufficiently high level in the chosen plant to allow for the practical oral delivery of a sufficient antigen dose to induce immune response. Many species can be adopted for production of recombinant vaccines, with tobacco being the most widely used host plant to date (Habibi and Zibaee, 2013). The advantages of the leafy crop tobacco include the high biomass yield, the ease of stable transformation either by cocultivation with *Agrobacterium tumefaciens* (Dugdale et al., 2014) or transiently by infiltration with transgenic agrobacteria (Leckie et al., 2011) or transfection with viral vectors (Rybicki et al., 2014). Another benefit is that tobacco is not used as a food crop, ensuring that a transformed line expressing a highly potent drug will not contaminate food resources. Other examples of leafy crops used for production of recombinant vaccines include alfalfa (Wigdorovitz et al., 1999), white clover (Lee et al., 2001), spinach (Yusibov et al., 2002; Karasev et al., 2005), lettuce (Kapusta et al., 2001), etc.

Tomato (*Solanumlycopersicum*) is an example of garden crops whose fruits are used for vaccine production. Tomato possesses a high fruit biomass yield and offers other advantages in terms of containment, because the plant is often grown in greenhouses. The most widespread use of tomato fruits in molecular farming has been in the expression of vaccine candidates. The first report on production of recombinant vaccines in tomato was the expression of rabies surface glycoprotein, which achieved the relatively high expression level (McGarvey et al., 1995). Other examples include cholera toxin B subunit (Jani et al., 2002) respiratory syncytial virus-F protein (Sandhu et al., 2000), toxin co-regulated pilus subunit A (TCPA) of *Vibrio cholera* (Sharma et al., 2008), as well as others. Other examples of fruits and vegetables used for antigen expression include potato (Mason et al., 1998; Yu et al., 2001), lettuce (Dong et al., 2014), carrot (Mendoza et al., 2011), etc.

Seed crops including both cereals (maize, rice, wheat and barley) and the grain legumes (soybean, pea, pigeon pea and peanut) have been used as ideal plant systems for production of recombinant vaccines (salaet al., 2003). The main advantage of seed crops is that recombinant proteins can be directed to accumulate specifically in the desiccated seed which is a natural storage organ, with the optimal biochemical environment for the accumulation of large amounts of protein. Moreover, recombinant proteins expressed in seeds have been shown to remain stable and active after storage at room temperature for over three years. Finally, seed proteome is fairly simple, which reduces the likelihood that contaminating proteins

will co-purify with the recombinant protein during downstream processing (Lamphear et al., 2004). According to Stoger et al (2000), Several factors should be considered when choosing an appropriate seed expression host, including geographical considerations, the ease of transformation and regeneration, the annual yield of seed per hectare, the yield of recombinant protein per kilogram of seed, the production cost of the crop, the percentage of the seed that is made up of protein and, inevitably, intellectual property issues.

Green microalgae have also emerged as new cell factories for production of recombinant vaccines. Microalgae possess advantages of prokaryote and eukaryote organisms simultaneously. On one side, they are unicellular organisms with very fast growth which facilitates mass production in short time (prokaryotic feature). On the other side, they are eukaryote and are able to process long eukaryotic peptides with accurate folding and appropriate post transcriptional modification (Specht et al., 2010). Examples of recombinant vaccine produced in microalgae include expression of Food and Mouth Disease Virus (FMDV) VP1 antigen in *Chlamydomonas reinhardtii* (Habibi et al., 2014), fusion protein containing the VP1 gene and the cholera toxin Bsubunit (Sun et al., 2003) and syndrome virus protein 28(VP28) (Surzycki et al., 2009) in chloroplast genome of the same species.

Several issues should be considered when selecting a plant species as an antigen expression host. The first issue is the form of vaccine delivery. Foreign proteins can be expressed in fresh tissue, such as mature plant leaves and germinating seedlings or in dry tissue, such as the seeds of cereals (Streatfield et al., 2001). Hydroponic culture is another ideal platform because the system makes it possible to secrete the expressed protein into the surrounding medium (Borisjuk et al., 1999). The plant species selected as expression system should possess optimum antigen expression, allows for cost-effective production, and can be manufactured into a practical form for oral delivery.

Plant transformation strategies

In general, recombinant subunit vaccines can be produced in plants either by stable or transient transformation. Stable transformation is the most common method widely practiced for production of transgenic lines expressing the antigen of interest. In this approach, the gene of interest is integrated in nuclear or plastid genome using biolistic or *Agrobacterium* mediated transformation methods. In *Agrobacterium* mediated gene transfer, the gene

of interest is inserted into the T-region of a disarmed Ti plasmid of *Agrobacterium tumefaciens*. The recombinant DNA is placed into *Agrobacterium*; a plant pathogen which is co-cultured with the plant cells or tissues to be transformed. The main disadvantage of this method is that it gives low yield and the process is slow. This method works especially well for dicotyledon plants like potato, tomato and tobacco. In this manner, the foreign antigen is stably inherited through successive generations (Lal et al., 2007). Some agronomically important plant species (e. g. most cereal crops)are recalcitrant to *Agrobacterium* transformation, and a biolistic method is frequently used for these plants (Awaleet al., 2012). In this approach, DNA coated gold particles are propelled into plant cells using compressed helium gas and becomes incorporated into chromosomal DNA. The biolistic method usually results in higher-copy-number plants compared to those generated by *Agrobacterium*, which can enhance expression. However, excessive copy numbers or very high-level expression of nuclear genes can cause gene silencing, resulting in low protein accumulation. Thus it is important to select transgenic lines that carry only between one and three copies of the transgene (Sala et al., 2003).

In transient transformation technique, the epitope of interest is engineered into a plant virus, usually within the coat protein gene. Infection of target plant by this viral vector results in intracellular production and accumulation of the epitope. The epitope sequence, as well as the viral genome, never become integrated into the plant genome and hence are only expressed by the generation of infected cells (Yusibov et al., 2002). This approach has been successfully applied to tobacco, black-eyed beans and spinach (Dalsgaard et al., 1997).The potential advantage of viral expression systems compared to stable plant transformation is that viral replication can greatly amplify the template for protein synthesis resulting in high-level protein accumulation (Pniewski, 2014).Transient transformation can also be achieved by *A. tumefaciens*. This method, called "agroinfiltration", involves the injection or vacuum infiltration of plants parts with a suspension of bacteria harboring the antigen of interest. This approach has a wide spectrum of applications and has been used for the study of molecular processes and production of interesting molecules of monoclonal antibodies (Orzaez et al., 2006), antigens of human (Mett et al., 2008) and livestock (Habibi et al., 2014) pathogens.

A newly developed transformation approach called

Magnifection is being used to overcome the limitations possessed by early platforms. It combines the two technologies namely agroinfiltration method and Tobacco Mosaic Virus (TMV)- based viral vectors system. This new approach allows the scalable production of a desired protein with high expression level and yield, low up- and downstream costs, reduced time, and most of all, reduced biosafety concerns (Gleba et al., 2005).

Enhancing antigen expression level in plants
Despite considerable advantages of green plants as feasible platforms for production of recombinant vaccine, low level of transgene expression is still a main drawback hindering commercial application of plant systems (Kang et al., 2003).As remarked by Habibi-Pirkoohi and Zibaei (2013), enhancing transgene expression in plant tissue will be a milestone in production of plant-based recombinant vaccines (Habibi-Pirkoohi and Zibaei, 2013).

To achieve this, several approaches have been proposed such as codon optimization, the use of strong plant promoters and untranslated leader sequences (Chikwamba et al., 2002).

Codon optimization is an efficient way to enhance transgene expression level in transgenic plants as different organisms prefer different codons when making a functional protein (Jabeen et al., 2010). It has been reported that codon optimization can enhance expression level in nuclear transformation as high as 5-fold (Fuhrmann et al., 1999) or up to 80-fold in chloroplast transformation (Franklinand Mayfield, 2005). Moreover, existence of rare codons in some organisms significantly reduces translation efficiency in transgenic plant (Gustafsson et al., 2004). Thus it is not surprising that many investigators make use of synthetic gene with optimized codon sequence (Habibi et al., 2014; Kang et al., 2004).

Presence of leader sequence at 5′ untranslated region is also efficacious in enhancing expression level. The prominent Kozak leader sequence (GCCACC) is a ribosome binding site (RBS) whose role in promotion of translation efficiency is well documented (De Angioletti et al., 2004). The upstream leader of Tobacco Mosaic Virus (TMV) called Ω sequence is another untranslated region which plays as a translational enhancer in higher plants. The CAA region residing within Ω sequence is responsible for translational enhancement and acts as a binding site for HSP101 heat shock protein, with the latter is necessary for translation improvement (Gallie, 2002).

In some cases, signal peptides such as SEKDEL sequence have been used to target the antigen in to endoplasmic reticulum (ER), where necessary enzymes and cellular machinery for proper folding are present(Xu et al., 2011). By addition of ER signals to transgene, high level of antigen expression has been observed in a number of studies (Kang et al., 2004; Haq et al., 1995; He et al., 2012). The ER signals are often attached to 3′ end of the transgene just before stop codon(Habibi et al., 2014).

Chloroplast transformation is an effective way to improve foreign antigen accumulation in plant tissues. This approach- usually referred to as cpDNA transformation- is based on the integration of the transgene into the circular chloroplast DNA (cpDNA) that is present in multiple copies in plant cells. Advantages of chloroplast engineering are numerous: the cpDNA molecule is completely sequenced in a number of important plants and is present to up to 10.000 copies per cell. Moreover, it has been shown that chloroplasts can properly process eukaryotic proteins, including correct folding and disulfide bridges (Daniel et al., 2001). Integration into cpDNA has two important advantages, the first being the foreign sequence is targeted to a precise cpDNA site by homologous recombination. This eliminates variability in gene expression and gene silencing, which often occurs in nuclear transformation. The second advantage is the enhanced accumulation of the recombinant antigen. Accumulation of recombinant protein in chloroplast engineering is far more than that of nuclear transformation (Ruhlman et al., 2010).

Oral delivery and mucosal immunity
The majority of infectious agents enter the body through mucosal membranes. Induction of mucosal immunity is best achieved by direct vaccine delivery to mucosal surfaces (Carter and Langridge, 2002). Orally delivered, non-replicating subunit vaccines have not yet achieved commercial success using any means of manufacture. The main hurdle facing the use of orally delivered immunogenic proteins is the likelihood that some proteins will be degraded after ingestion and that some immunogens may not be recognized efficiently at mucosal immune effect or sites in the gut. Although this is a potential limitation, the use of plants as a protein biomanufacturing system offers advantages in that the cost of obtaining the end product is comparatively low. Plant-derived vaccines have demonstrated the ability to induce both systemic and mucosal immune responses (Kong et al., 2001). The major obstacle to oral vaccination is the digestion of the antigenic protein in the stomach.

Vaccines derived from plant cells have been shown to overcome this problem through the protective effect of the plant cell wall. Like liposomes and microcapsules, the plant cell wall allows gradual release of the antigen onto the vast surface area of the lower digestive tract (Streatfield, 2006).

Diseases targeted for recombinant plant based vaccines

A large number of diseases have been studied for production of recombinant vaccines. This includes both human and animal infectious diseases and various plant species have been investigated as the host for production of recombinant vaccines. Foot and Mouth Disease (FMD) and hepatitis B are two example of disease for which many investigations have been carried out in trying to obtain an effective plant-based recombinant vaccine. A summary of plant-derived recombinant vaccines is presented in table 1. The table shows that *Agrobacterium*-mediated nuclear transformation is the dominant procedure for achieving transgenic plants and tobacco, potato and tomato are among the species widely used for recombinant vaccine production. The long list of recombinant vaccines is undoubtedly is not limited to the examples presented in this review, and the list is growing fast by introducing new vaccines, more sophisticated gene construct designs and application of new plant species.

Safety issues, Public acceptance and Commercialization

Plant-derived vaccines are free from human and animal pathogen contaminants. Furthermore plant DNA is not known to interact with the animal DNA and plant viral recombinants do not invade mammalian cells. Nevertheless, some concerns need to be addressed before recombinant vaccines release in market. One of the fears is that GM-pollen may escape to the nature and bear harmful influences on biodiversity. To address this concern, some pollen containment approaches have been developed which are often based on establishment of different forms of male sterility (Sala et al., 2003).

Analternative way of solving the problem is engineering vaccines in to the cpDNA, which is not transmitted to the sexual progeny through the pollen grains (Daniel et al., 2011). With this approach, land needed for industrial plant-derived vaccine-production will be in the order of a few thousand square meters because expression level of the antigen is of high magnitude. This enables vaccine-producing transgenic plants to be set apart

from field grown crop plants. Another public concern in GM-plants is the presence of antibiotic resistance genes (used as selective marker in most transgenic plants). Approaches have now been developed to generate GM-plants (with both nuclear or cpDNA integration)that do not carry these genes (Puchta et al., 2000).

Table 1. Recombinant vaccines produced in transgenic plants

Antigen	Transformation method	Plant host	Reference
E. coli heat labile enterotoxin (LT-B)	*Agrobacterium*-mediated	Potato	(Mason et al., 1998)
E. coli heat labile enterotoxin (LT-B)	*Agrobacterium*-mediated	Tomato	(Walmsley et al., 2003)
E. coli heat labile enterotoxin (LT-B)	*Agrobacterium*-mediated	Soybean	(Moravec et al., 2007)
Foot and Mouth Disease antigen VP1	*Agrobacterium*-mediated	Alfalfa	(Wigdorovitz et al., 1999)
Foot and Mouth Disease antigen VP1	Chloroplast transformation (biolistic)	Tobacco	(Li et al., 2006)
Foot and Mouth Disease antigen VP1	Agroinfiltration	Tobacco	(Habibi et al., 2014)
Foot and Mouth Disease antigen VP1	*Agrobacterium*-mediated	Microalgae (*C. reinhardtii*)	(Habibi et al., 2014)
Hepatitis B antigen HBsAg	*Agrobacterium*-mediated	Potato	(Kong et al., 2001)
Hepatitis B antigen HBsAg	*Agrobacterium*-mediated	Lettuce	(Koprowski et al., 2001)
Hepatitis B antigen HBsAg	*Agrobacterium*-mediated	Lupin (*Lupinusluteus*L.)	(Kapusta et al., 1999)
HIV glycoprotein	*Agrobacterium*-mediated	Spinach	(Karasev et al., 2005)
HIV antigen p24	*Agrobacterium*-mediated	Potato	(Obregon et al., 2006)
Rabies G and N proteins	*Agrobacterium*-mediated	Tobacco	(Yusibov et al., 2002)
Rinderpest virus hemagglutinin (H)	*Agrobacterium*-mediated	Tobacco	(Khandelwal et al., 2003)
Newcastle disease F antigen	biolistic	Maize	(Guerrero-Andre et al., 2006)
Respiratory Syncytial Virus (RSV) antigens F and G	*Agrobacterium*-mediated	Tomato	(Sandhu et al., 2000)

Despite numerous advantages of plant-based recombinant vaccines, none of the major pharmaceutical companies is directing funding towards the development of plant-derived vaccines for infectious diseases. This reluctance about commercial production of plant-based recombinant vaccines is mainly due to concern about the potential for significant return on investment; uncertainties in the regulatory processes; limited human clinical trial data that establish required dosages, timing of delivery, and evaluation of possible adverse immunological effects; and finally, a lack of personnel with sufficient expertise in plant biology (Zhang et al., 2011).Participation of both the public sector and the non-profit sector will be essential to provide leadership and investment support to unlock the potential of plant-derived vaccines.

Conclusion

Application of green plants for production of recombinant vaccines offers many advantages over traditional methods making this approach a practical way for manufacture of mucosal vaccines on a global scale. Since the pioneer work of Curtiss and Cardineau (1990), many vaccine antigens have been expressed in different plant species to demonstrate the feasibility of oral plant-based vaccines. Despite the promising future and several successes achieved in this field, different issues will have to be established and well defined such as high expression levels, product quality, downstream process costs, regulatory framework, efficacy and safety. Moreover, a large part of the researches in the field of recombinant vaccine production are carried out in tobacco which is not an edible plant and, due to possessing high level of alkaloids, is not affordable as an oral vaccine. Thus, it is necessary to try other plants such as fruits and vegetables to realize production of a plat-based recombinant vaccine.

Growing progress in the field of biotechnology and plant genetic engineering will undoubtedly assist in improvement of plant-based recombinant vaccines.

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