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The Editorial

Cancer, A Big Monster, Which Should Be Defeated / The Editorial

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Abstract

Cancer remains a major cause of death worldwide. Huge research and identification of several markers have resulted in better understanding of its mechanism. Researches focusing on cancer stem cells and their role in metastasis will help the scientific community to propose the therapeutic approaches for treatment of this monstrous disease. Interest of governmental agencies and inter-communications of molecular biologists with clinicians can boost the new ideas in identification and characterizations of cancer stem cells. It will also help to elucidate their roles in tumor progression and hopefully would result in better ways to reduce the mortality related to cancer.

***Keywords*:** Cancer stem cells, metastasis, abnormal growth

The term "Cancer" is used for abnormal growth of cells in different organs, which can metastasize to other parts of the body. Although there has been much progress to unravel the mechanisms involved in this type of diseases, and also the progress in treatment of certain types of cancers, we are still far from finding specific and effective treatments and cancer remains a major cause of death worldwide. In Iran cancer is the third cause of death after coronary heart diseases and accidents. However, its mortality is on the rise due to increase in life expectancy and westernized lifestyle (Mousave, et al., 2008). So it is very important for us to establish better screening and diagnosis systems, as well as more effective approaches for treatment of cancer.

Research in the past decade has shown that "cancer stem cells" with self-renewal and multilineage differentiation potential (Vermeulen, et al., 2008) are responsible for the growth and relapse of many tumors. The balance between cancer stem cells and the differentiated cells in a tumor might be dependent on the tumor and its genetic background (Medema, 2013).

Since genetic background is important in both survival and metastasis of cancers (Hunter, et al., 2003; Lindström, et al., 2009) and the incidence of cancers is also different in various parts of the world, it is very important for us to focus more on samples from Iranian patients to derive cancer cell lines, isolate cancer stem cells and study their gene expression profiles, elucidate the mechanisms involved in their proliferation and differentiation and also to find better ways to fight this disease based on personalized medicine. To reach this goal, improved national funding for cancer research and also a better interaction and collaboration between scientists working in basic science and clinicians are required.

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Research Article

Isolation, Culture and Characterization of Chicken Primordial Germ Cells

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Abstract

Nowadays, production of recombinant proteins in eukaryotes is gaining good deal of attention. Transgenic chicken as a eukaryotic system has a high potential for producing recombinant proteins. Post-translational changes, especially glycosylation, are characteristic of the eukaryotic proteins. In practice we need to choose a proper expressing host when considering over-expression of a recombinant protein. Chickens are among the well-considered candidates for such application. Production of transgenic chickens could be achieved in different ways, including application of primordial germ cells. Primordial germ cells are progenitor of sperm and ovum. These cells are round, with a big nucleus and a cytoplasm with lipid and glycogen particles. The first step for having transgenic chickens is isolation and culture of the primordial gem cells. In the present study, these cells were isolated by centrifugation method in presence of ficoll and using magnetic cell sorting, and were cultured in optimal culture medium. These cells were finally characterized with defined methods, like Periodic acid-schiff staining, alkaline phosphates activity assessment, and antibody staining.

***Keywords*:** primordial germ cells, transgenic chicken, recombinant proteins

Introduction

Scientific studies in the field of producing transgenic animals have progressed significantly as a model of various human diseases and for the purpose of producing recombinant drugs. Gordon produced the first transgenic mouse by introducing gene into pronuclear fertilized egg (Gordon et al., 1980). Producing transgenic chickens was also started from 1980, and first transgenic chicken was produced with the help of viral vectors and injecting them to the blastoderm cells (Petitte et al., 1990). Transgenic chickens have many priorities in comparison with other animals, and these capabilities have attracted the attention of many researchers around the world. Different methods have been used for producing transgenic chickens (Houdebine, 2002; Love et al., 1994; van de Lavoir et al., 2006). Nevertheless, primordial germ cells are known as the best option for producing transgenic chickens, because separating these cells is almost easy, they have a high germ line transmission, the ability to keep these cells in culture medium for genetic manipulation, and keeping these cells in culture medium does not reduce germ line transmission rate (Han, 2009).

Primordial germ cells (PGCs) are actually precursors of sperm and ovum. These cells were first described by Swift (Swift, 1915). These cells are round, have a big nucleus and a cytoplasm with lipid and glycogen granules. At first, around 50 cells were isolated from epiblast and move toward head fold to be located in germinal crescent. After forming embryo cardiovascular system, these cells migrated toward genital ridge through blood and are finally located in primitive gonad (Kunwar et al., 2006). This type of PGCs migration is specific to the poultry, and is different from mammals. Specific route of chicken primordial germ cells has made their isolation available in different points including from germinal crescent, blood, and

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primitive gonad (Fujimoto et al., 1976). In the present study, primordial germ cells were isolated from blood by centrifuge in the presence of ficoll at stage 14 HH (Hamburger and Hamilton) , and were isolated from primitive gonad using magnetic cell sorting (MACS) at stage 28 HH, then by optimizing growth conditions in culture medium which was done by presence of feeder cells and using growth factors, these cells were cultured in laboratory. After being sorted, these cells were characterized; using specific methods; like Periodic acid-Schiff staining (PAS) staining, alkaline phosphates (AP) staining, and specific antibodies. To the best of my knowledge, this is the first report of isolation and characterization of primordial germ cells in Iran.

Materials and Methods

Chicken embryos

Fertilized chicken (*Gallus gallus domesticus*, Ross breed) eggs were obtained from a commercial hatchery and incubated in a rotary egg incubator (DORNA System, Iran) at 38°C at 60% humidity with rocking at an angle of 90 degree every 90 minutes.

Isolation of primordial germ cells

Isolation with Ficoll

Under a laminar hood, the blunt end of the eggs was cut horizontally with a diamond cutter. After checking that embryos had developed to the Hamburger–Hamilton stage 14 HH (Hamburger and Hamilton, 1992), blood was collected from 30 embryos and placed in Dulbecco's minimal essential medium (DMEM; Biosera, UK) containing 10% fetal bovine serum (FBS, Invitrogen, USA). After centrifugation at 800xg for 10 minutes, pellet of blood cells was resuspended in 16% Ficoll-400 (Sigma-Aldrich Corp., Germany), was covered with 6.3% Ficoll-400 solution, and was centrifuged at 800xg for 30 minutes (Yasuda et al., 1992). Primordial germ cells were collected at the interface between the 16% and 6.3% Ficoll phases. Ficoll was removed by centrifuging the samples twice at 200xg for 8 minutes.

Isolation with magnetic cell sorting (MACS)

From eggs incubated for 5.5 days (stage 28) (Hamburger and Hamilton, 1992), embryos were removed and rinsed three times with calcium-free phosphate buffered saline (PBS). Using fine glass needle under a stereomicroscope, the genital ridge (with its mesonephrus attached) was dissected out. Then gonadal tissue was dissociated into cells in a solution of 0.25% trypsin/0.02% EDTA (Biosera, UK) at 37°C for 10 minutes. Dissociated cells were placed in DMEM containing 10% FBS. The dispersed cells were filtered through a 100-µm mesh, and then washed with PBS and centrifugated at 300xg for 10 minutes. To isolate primordial germ cells, gonadal cells were incubated with anti-stage specific embryo antigen (anti-SSEA)-1 microBeads (Miltenyi Biotech, Germany) in 20 µl mixed with 80 µl of Buffer (PBS supplemented with 0.5% BSA and 2mM EDTA) for 15 minutes at 4°C. Cells were washed with 1 ml of buffer, and were resuspended in 200 µl of buffer and subsequently loaded onto MACS columns (Kim et al., 2004). Using the magnetic field of a MACS separator, PGC cells bound to anti-SSEA-1 microbeads were isolated and were cultured in Knockout DMEM (KO-DMEM; Invitrogen, USA) medium.

Short-term culture of primordial germ cells

PGC cells are sensitive cells and need a specific culture medium for growth. This appropriate medium was optimized after a while, as these cells have the ability of growth in this medium. Primordial germ cells were seeded in 24 well cell culture plate with or without the presence of mitotically inactivated STO cells (3×10^4 cells/cm^2) in KO-DMEM, which was supplemented with 7.5-10% (vol/vol) FBS, 2.5-5% (vol/vol) chicken serum (Sigma-Aldrich, USA), 1× Penicillin (100 U/ml)/Streptomycin(100 µg/ml) (Biosera, UK) , 2mM glutamine (Sigma-Aldrich, USA), 1mM pyruvate (Sigma-Aldrich, USA), 10 mM nonessential amino acids (Sigma-Aldrich, USA), 10 mM HEPES (Sigma-Aldrich), 0.15 mM β-mercaptoethanol (Sigma-Aldrich, USA), 2 ng/ml mouse leukemia inhibitory factor (LIF) (Sigma-Aldrich, USA), 3-12 ng/ml human basic fibroblast growth factor (Sigma-Aldrich, USA), and 6-10 ng/ml stem cell factor (Sigma-Aldrich, USA). The seeded cells were then cultured in a CO_2 incubator at 37.5°C in an atmosphere of 5% CO2 in air with 90% relative humidity. Half of the culture medium was replaced with new culture medium every two days, and the old culture medium was replaced totally after one week.

Periodic acid-Schiff (PAS) staining

Isolated PGCs and PGC colonies were fixed in 4% paraformaldehyde (PFA; Electron microscopy sciences, USA) in PBS (vol/vol) for 10 minutes. After rinsing in PBS, the cells were then immersed in periodic acid solution (Sigma-Aldrich, USA) for 5 minutes, and subsequently were treated with

Schiff's reagent (Sigma-Aldrich, USA) for 15 minutes. All procedures were performed at room temperature, and the stained PGCs were observed under a white-light microscope (Olympus BX51, Japan).

Alkaline phosphates staining

After fixation with 4% PFA for 10 minutes, cells and colonies were washed three times with alkaline phosphatase (AP) buffer, and then covered with AP solution (1ml of for each coverslip). After 15 minutes, cover slips were washed with AP buffer and were observed under a microscope (Olympus BX51, Japan). The AP buffer contained 100mM Tris-HCl (Merck, USA), 100mM NaCl (Merck, USA), 5mM $MgCl_2$ (Merck USA), 0.05% Tween 20 (Merck, USA), with a pH of 9.5. AP solution was prepared by mixing 120µl of 1% BCIP (5-bromo-4-chloro-3-indolyl phosphate; Fermentas, USA) in 100% DMF (dimethylformamide; Merck, USA), 120µl of 1.5% NBT (nitro blue tetrazolium; Fermentas, USA) in 70% DMF, and 5 ml of AP buffer.

Immunocytochemistry

Isolated PGCs and PGC colonies were fixed with 4% PFA for 10 minutes. After washing with PBS (three times, five minutes each), cells were permeabilized with 0.5% Triton X-100 (Merck, USA) for 5 minutes. After washing with PBS, to minimize nonspecific binding of antibodies, the fixed cells were blocked for 45 minutes with a blocking buffer consisting PBS with 5% (v/v) BSA. Then, cells were washed three times with PBS, and were incubated with each of the primary antibodies including mouse anti-SSEA-1 (1:100; Santa Cruz Biotechnology Inc., USA), mouse anti-SSEA-4 (1:150; Santa Cruz Biotechnology Inc., USA), rabbit anti-VASA (1:200; Santa Cruz Biotechnology Inc., USA), After incubation for 2 hours in the primary antibody solution in a humid chamber at room temperature, the cells were washed three times with PBS. Then, cells were incubated with either of the secondary antibodies, donkey anti-rabbit IgG conjugated to Cy3 (Jackson ImmunoResearch, USA), donkey anti-rabbit IgG conjugated to HRP (Jackson ImmunoResearch, USA), donkey anti-mouse IgM conjugated to HRP (Jackson ImmunoResearch, USA), , and goat anti-mouse IgG conjugated to HRP (Dianova, Germany), in a dark humid chamber for 1 hour at room temperature. After washing with PBS, coverslips were mounted on the slide with the application of 40 µl antifade plus DAPI (DENAzist Asia, Iran) or DAB staining (Fermentas, USA) and analyzed under a fluorescence or light microscope (Olympus BX-UCB, Japan). Negative controls, without the use of primary antibody, were only stained with the secondary antibody.

Results

Characterization of primordial germ cells

The isolated PGCs (from HH stages 18 and 28) contained a large nucleus and many prominent vacuoles in the cytoplasm (Fig. 1A). PAS (periodic acid-Schiff) staining of PGCs produced a diffuse staining pattern throughout the cytoplasm indicating a cytoplasm rich in glycogen particles (Fig. 1B). Staining with alkaline phosphatase activity, a pluripotency cell marker, also was positive for primordial germ cells (Fig. 1C). Many antibodies are used for showing the cell epitopes of primordial germ cells. For example, in human we can refer to SSEA-3 ،SSEA-4 and TRA-1 (Henderson et al., 2002), in mouse, SSEA-1 is recognized as the pluripotency marker (Resnick et al., 1992). In chicken, different markers like SSEA-1 ،SSEA-3 ،SSEA-4 and EMA-1 has been reported (Jung et al., 2005). In the present study, Immunostaining with the pluripotency markers, SSEA-1 (stage-specific embryonic antigen 1) and SSEA-4 (stage-specific embryonic antigen 4), demonstrated that PGCs were strongly stained for these markers (Fig. 1D, 1E). In addition, to determine whether isolated PGCs also express the germ cell-specific protein, we used immunofluorescence to detect VASA (CVH), a RNA processing protein important for germ cell survival and specification (Kuramochi-Miyagawa et al., 2010). Immunostaining with an antibody to VASA illustrated that in Primodial germ cells it was localized throughout the cytoplasm (Fig. 1F). This is consistent with the reported cytoplasmic localization of CVH in avian germ cells (Tsunekawa et al., 2000).

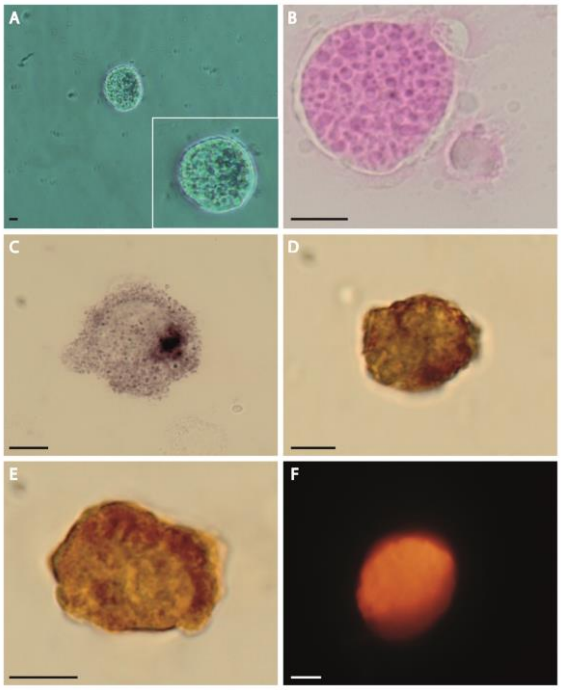

Figure 1. Characterization of PGCs. A) A single PGC under white light microscope, B) A single PGC Stained with PAS, C) A single PGC stained for AP activity, D) A single PGC immunostained for SSEA-1, E) A single PGC immunostained for SSEA-4, F) A single PGC immunostained for VASA.
Scale bar represents 5µm.

Short-term culture of primordial germ cells

A number of experiments were carried out to identify the most appropriate concentration of sera and growth factors for in vitro culture of PGCs. The best results obtained with STO as feeder cells, 10

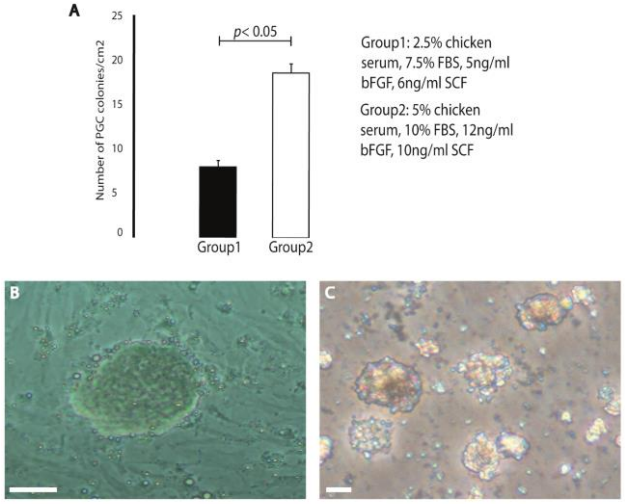

Figure 2. Culture of primordial germ cells. A) Optimization culture medium for PGCs . B) A single colony under white light microscope after 2 weeks. C) Apoptosis of the colonies occurred in the absence of the growth factors (Scale bar represents 50µm).

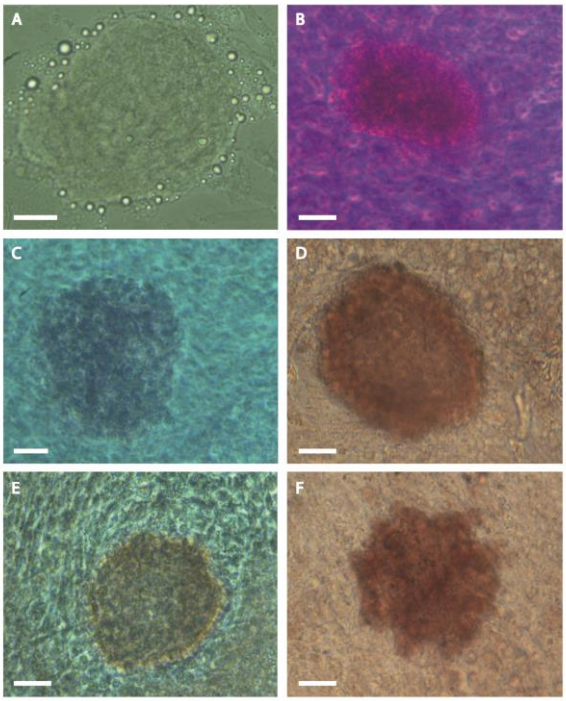

Figure 3. Characterization of colonies of PGCs. A) A single colony under white light microscope, B) A single colony stained with PAS, C) A single colony stained for AP activity, D) A single colony immunostained for SSEA-1, E) A single colony immunostained for SSEA-4, F) A single colony immunostained for VASA (Scale bar represents 25µm).

Percent FBS, 5 percent chicken serum, 12ng/ml of human basic fibroblast growth factor, and 10ng/ml of stem cell factor (Fig 2A). Typical colonies of PGCs were observed two weeks after culture (Fig. 2B). Apoptosis of the colonies occurred even in the absence of the growths factor (Fig 2C). Cell colonies resembling ES colonies were formed, with the appearance of discs with smooth ridges and densely packed cells or multilayer congregates. Primordial germ cells form colony after 7 to 10 days. These colonies were adhered to the surface of STO cells. In the present study, they had a circular appearance with a specific margin (Fig 2B).

Characterization of colonies

Colonies of primordial germ cells (Fig 3A) also were characterized with specific methods. These colonies were positive with periodic acid Schiff (Fig 3B) and alkaline phosphatase (Fig 3C). Staining with pluripotency markers, SSEA-1 (Fig 3D) and SSEA-4 (Fig 3E) were positive. To confirm the germ-cell identity of the colonies, we detected the vasa which has been shown to be a specific germ-cell marker (Fig 3F).

Discussion

Primordial germ cells and the colonies obtained from PGCs were characterized by staining with PAS, AP, and specific antibodies. Primordial germ cells were cultured under different conditions; in the presence and absence of feeder cells, growth factors and using simple or enrichment culture medium. But as primordial germ cells are sensitive to environmental conditions, they need enrichment culture, growth factors, and feeder cells for growth. Three factors are more important than others e.g. SCF, bFGF, and LIF. In zebrafish, epidermal growth factor, bFGF, kit ligand-a, and stromal cell derived factor-1b were used (Fan et al., 2008). Also, in mouse and human bFG, SCF, and LIF have been used mainly for growing PGCs (Matsui et al., 1992; Shamblott et al., 1998).

In the present study, these factors were added to the culture medium for growing primordial germ cells. The best growth conditions for PGCs are provided in the presence of three factors. Fibroblastic growth factor is activating some signal pathways like MEK/ERK signal, or changing the expression of some genes like IL17RD, also LIF and SCF factors are stopping apoptosis pathways through controlling the expression of tTGase and inhibiting the fragmentation of DNA so these factors play an important role in survival and proliferation of primordial germ cells (Choi et al., 2010; Pesce et al., 1993). Presence of STO feeder cells is important in the culture medium from two aspects; first, these cells provide factors and nutrition for growth of PGCs, and second, a physical connection is made between PGCs and STO cells which help the growth and maintenance of primordial germ cells *in vitro*. It has been shown that physical connection between PGCs and surrounding cells are important in aggregation of these cells in genital ridge (Gomperts et al., 1994) and it's similar for colony formation in culture medium.

Providing an optimized medium to grow PGCs in culture is very important, because a significant amount of primordial germ cells is needed for producing transgenic chickens, and as the number of isolated cells is low in each separation, significant amount can be achieved by culturing and proliferate them in optimized culture medium. On the other hand, by the presence of primordial germ cells in culture medium, it is easier to create changes in genome of primordial germ cells. After confirming the gene transfer into the genome of these cells, injecting these transformed cells into the embryo is done to get the transgenic chicken with the desire capability.

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Research Article

Evaluation of relationship between HNF-1α and GLP-1R polymorphisms and type 2 diabetes in a population living in northeast of Iran

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Abstract

The prevalence of type 2 diabetes mellitus (T2DM) is rising dramatically in the Middle East, especially in the Islamic Republic of Iran, but the genetic basis of type 2 diabetes in Iran is poorly understood. Polymorphisms of hepatocyte nuclear factor-1α (HNF-1α) and glucagon-like peptide-1 receptor (GLP-1R) genes showed association with type 2 diabetes in several ethnic groups. In this study, we evaluated whether these markers confer susceptibility to T2DM in a diabetic population living in Mashhad (northeast of Iran). Genotyping of Ala98Val (HNF-1α) and Thr149Met (GLP-1R) was done by the restriction fragment length polymorphism-PCR (RFLP-PCR) method in the following groups: 1) early-onset diabetes (age at onset ≤ 35 years); 2) late-onset diabetes (age at onset > 35 years); and 3) control. Our results showed that CT (Ala/Val) genotype of HNF-1α was higher in the early-onset type 2 diabetic group compared to the controls but difference was not significant. We did not find the GLP-1R Thr149Met mutation in all participants. The prevalence of the HNF-1α (Ala98Val) and (GLP-1R) Thr149Met mutations has not been previously reported in Iranian participants. We conclude that these mutations are not a common cause of T2DM in our studied population.

***Keywords*:** Type 2 diabetes; Hepatocyte nuclear factor-1α; Glucagon-like peptide-1 receptor; Polymorphism

Introduction

Type 2 diabetes is a disease with a rising prevalence worldwide (Gadsby, 2002). The prevalence of diabetes in Iran ranges from 1.3% in rural areas to 14.5% in large cities and most patients have type 2 diabetes (Azizi et al., 2003a; Azizi et al., 2003b). It is predicted that the developing countries will contribute 77.6% of the total number of diabetic patients in the world by the year 2030 (Azizi et al., 2003b; Hussain et al., 2007). In addition to a sedentary lifestyle related to recent affluence leading to obesity and insulin resistance, the rising prevalence has been attributed to genetic predisposition (Bener et al., 2005; Yaturu et al., 2005). Impairment in insulin secretion plays a major role in the pathogenesis of type 2 diabetes in addition to insulin resistance (Association, 2009). Therefore, genetic variants in insulin secretion pathways are plausible candidate genes for type 2 diabetes. In the present study, single nucleotide polymorphisms (SNPs) in the genes regulating insulin secretion (TCF1 [encoding HNF-1α], and GLP-1R) were evaluated as risk factors for type 2 diabetes mellitus in a diabetic population living in Mashhad (northeast of Iran).

The most frequent monogenic forms of T2DM with profound defect in insulin secretion is autosomal dominant diabetes and its early onset form is called MODY (maturity onset diabetes of the young). Mutations in the hepatocyte nuclear factor-1α (HNF-1α) cause the type 3 of MODY (MODY3). HNF-1α is an important transactivating factor that is involved in pancreatic B-cell glucose sensing because it increases the transcription of many genes participating in the insulin secretion process (Bowden et al., 1997; Mahtani et al., 1996; Shaw et al., 1998). Common variations in this gene were associated with impaired insulin secretion and the risk of type 2 diabetes (Holmkvist et al., 2006; Urhammer et al., 1997). HNF-1α mutations are the most common mutations in Western and Asian countries (Ellard, 2000; Herman et al., 1994; Iwasaki et al., 1997; Lee et al., 2001).

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Previous reports in Danish Caucasians have shown that insulin responses to an oral glucose load have decreased in Val carriers of the HNF-1α Ala98Val polymorphism compared with the Ala homozygous individuals, suggesting that this amino acid replacement might influence β-cell function (Urhammer et al., 1997). Earlier studies in a Finnish population showed an association between Ala98Val polymorphism and T2DM with a prevalence of 13.2% (Lehto et al., 1999).

The gut hormone glucagon-like polypeptide-1 (GLP-1) is an incretin hormone that is released from epithelial cells of the gastrointestinal mucosa after food intake. Secreted GLP-1 binds to the GLP-1 receptor (GLP-1R) on the pancreatic β–cell and markedly increases glucose-dependent insulin secretion (Drucker, 2006). The GLP-1R is a candidate gene for diabetes mellitus, as mutations may induce the impaired insulin response that is a characteristic feature of non-insulin-dependent diabetes mellitus (NIDDM) (Zhang et al., 1994). Studies of the GLP-1R have been directed towards identifying polymorphisms in the GLP-1R gene that may be a contributing factor in the pathogenesis of diabetes. A GLP-1R polymorphism in which threonine 149 is substituted with a methionine residue has been identified in a patient with type 2 diabetes but was not found in non-diabetic control participants (Tokuyama et al., 2004). Expression of a nonsynonymous single nucleotide polymorphism, which results in substitution of methionine for threonine at position 149 of GLP-1R in cell systems, decreases binding affinity for glucagon-like peptide (GLP)-1 and intracellular signaling after hormone receptor binding (Beinborn et al., 2005). The Thr149Met substitution may alter the conformation of the binding pocket by indirect mechanisms, and thereby impair high-affinity interactions between the receptor and the amino-termini of cognate ligands. These functional effects suggest that genetic variation in GLP-1R may alter responsiveness to GLP-1 in vivo (Sathananthan et al., 2010).

No information is available on the prevalence of the HNF-1α Ala98Val and GLP-1R Thr149Met mutations among the Iranian population and their association with type 2 diabetes. The present study was undertaken to investigate the prevalence of these polymorphisms in a diabetic population admitted to the Shahid Ghodsi hygiene center, Mashhad, Iran.

Materials and Methods

Participants

This study was performed on 150 unrelated participants from the city of Mashhad (North east of Iran, Khorasan Razavi province) who were referred to Shahid Ghodsi hygiene center. Mutational analysis was performed in 50 healthy control participants (37 men and 13 women) with a mean body mass index (BMI) of 24.81 ± 3.37 kg/m^2 and 100 patients with type 2 diabetes mellitus whom were divided into two groups on according to the age at which diabetes were diagnosed. One group with early-onset diabetes ≤ 35 years included 41 patients (male/female: 12/29) with a mean BMI of 26.57 ± 2.94 kg/m^2 and the other group with late-onset diabetes > 35 years included 59 patients (male/female: 22/37) with a mean BMI of 26.47 ± 2.97 kg/m^2. Since MODY3 often takes place in youth, patients had been assigned to such division in order to compare patients with early-onset diabetes with those of late-onset diabetes. Before participation, the purpose and risks of the study were carefully explained and informed consents were obtained. The protocol was approved by the committee of ethics in Ferdowsi University of Mashhad.

DNA Analysis

Genomic DNA was extracted from peripheral blood leukocytes of T2DM patients and control participants using a genomic DNA purification kit (Ferments, Canada) and quantitated by ultra violet (UV) absorption at 260 nm.

Genotyping of polymorphism

HNF-1α Ala98Val and GLP-1R Thr149Met were genotyped by restriction fragment length polymorphism-polymerase chain reaction (RFLP-PCR) using the listed primers (Table 1).

Table 1. Primers for amplification of *HNF-1α* and *GLP-1R* exons.

Gene	Primers	Product size (bp)
HNF-1α	Fwd: CGTGGCCCTGTGGCAGCCGA Rvs: GGGCTCGTTAGGAGCTGAGGG	424
GLP-1R	Fwd: CTGCTTCATTCCTCTATCTGGG Rvs: TGTATTCACCTCTCTGGCCTTG	380

Research Article

Table 2. Association of HNF-1α Ala98Val polymorphism with type 2 diabetes.

Allele/genotype	Diabetic subjects: Early-onset Diabetes	Diabetic subjects: Late-onset Diabetes	Control subjects	OR (95% CI)	p
Number (n)	41	59	50		
C/C frequency% (n)	90.2 (37)	100 (59)	96 (48)	Reference	
C/T frequency% (n)	9.8 (4)	0 (0)	4 (2)	1 (0.13–11.42)	0.99
Allele C	95.1 (78)	100 (118)	98 (98)	Reference	
Allele T	4.9 (4)	0 (0)	2 (2)	1.96 (0.27–22.06)	0.68

Note: No subjects had the T/T genotype

In order to amplify HNF-1α gene, the 50 µl reaction mixture contained 0.2 µg of DNA, 100 µM of each dNTP, 2 U Taq DNA polymerase, 1.5 mM $MgCl_2$, and 10 pmol of each primer. The PCR conditions were 5 min at 94 ºC; 35 cycles for 30 s at 95 ºC, 15 s at 64 ºC, 3 min at 72 ºC with a final extension of 5 min (Lim et al., 2008). For GLP-1R, the 50 µl reaction mixture contained 0.2 µg of DNA, 200 µM of each dNTP, 2 U Taq DNA polymerase, 3 mM $MgCl_2$, and 10 pmol of each primer. PCR was performed as follows: DNA denaturation for 5 min at 94 ºC; 35 cycles of: 30 sec at 96 ºC, 15 sec at 60 ºC, 3 min at 72 ºC, and a final extension for 5 min at 72 ºC (Tokuyama et al., 2004). The fragments were amplified in a thermal cycler (Primus 96 advanced ® / Primus 96 advanced ® Gradient).

A 10 µl of PCR product was digested in a total volume of 25 µl with 2.5 U of restriction enzyme HaeIII (HNF-1α exon 1 polymorphism Ala98Val) or NlaIII (GLP-1R exon 5 polymorphism Thr149Met) in the buffer provided by the manufacturer (Fermentas GmbH, Germany) at 37 ºC for 4 and 6 hours, respectively. The expected sizes of HaeIII digestion products are as follows: the CC (Ala/Ala) genotype 61, 85, and 167 bp; the CT (Ala/Val) genotype 61, 85, 167, and 252 bp; and the TT (Val/Val) genotype 61 and 252 bp. For the GLP-1R PCR product, the sizes of the restriction fragments digested with NlaIII are as follows: the CC (Thr/Thr) genotype 207, 133, 29, and 11 bp; the CT (Thr/Met) genotype 207, 135, 133, 72, 29, and 11 bp; and the TT (Met/Met) genotype 135, 133, 72, 29, and 11 bp.

The PCR and restriction fragments were electrophoresed on 2% agarose gel and visualized by ethidium bromide staining in a gel documentation system (Kalagene Pajooh, Iran).

Biochemical Assays

Fasting blood samples were taken from the patients and control participants for the estimation of glucose and lipids. Serum glucose, triglyceride, cholesterol, LDL-cholesterol (LDL-C), and HDL-cholesterol (HDL-C) were determined by enzymatic colorimetric assays using standard kits (Pars Azmoon, Mashhad, Iran).

Statistical Analysis

Data was analyzed with the SPSS program (version 18.0). Results are given as mean ± S.D. or percentages. To compare quantitative data in groups of carriers of different genotypes, the unpaired Student's t-test was used. Odd ratios (OR) and 95% confidence intervals (CI) were used for estimating the strength of association between different groups and alleles or genotypes of HNF-1α and GLP-1R genes polymorphism. To provide separate ORs for each genotype, dummy variables were used, with a wild-type genotype used as a reference group. p- Values of less than 0.05 were considered significant.

The significance of interaction between clinical characteristics and polymorphic variants was assessed using a multivariate analysis of variance (MANOVA).

Table 3. Clinical and biochemical features of the studied population.

Characteristic	Early-onset diabetes (N=41)	Late-onset diabetes (N=59)	Control (N=50)
Male/Female	22/37	12/29	13/37
BMI (kg/m^2)	26.59±2.88	26.37±3.01	24.82±3.36
FBS (mg/dL)	203.73±60.12	175.59±62.31	87.90±9.60
Triglycerides (mg/dL)	156.04±46.54	155.13±54.71	106.51±36.73
Cholesterol (mg/dL)	202.60±42.64	198.13±34.88	174.20±39.01
LDL-C (mg/dL)	132.47±34.77	126.93±38.43	109.62±35.26
HDL-C (mg/dL)	36.60±5.80	40.45±7.22	41.84±10.05

BMI, Body mass index; FBS, Fasting plasma glucose; LDL-C, Low density lipoprotein-cholesterol; HDL, High density lipoprotein-cholesterol; Data are presented as means ± SEM.

Results

The results of investigating HNF-1α Ala98Val and GLP-1R Thr149Met polymorphisms are shown in Table 2. We have not detected restriction fragment length polymorphism in the exon five of human GLP-1R gene by NlaIII digestion. The Thr149Met mutation (ACG→ ATG) was absent in all patients and controls. By RFLP-PCR, we were able to clearly distinguish the 2 control participants and 4 patients of the early-onset T2DM group with HNF-1α Ala98Val (GCC→ GTC) mutation (Figure 1).

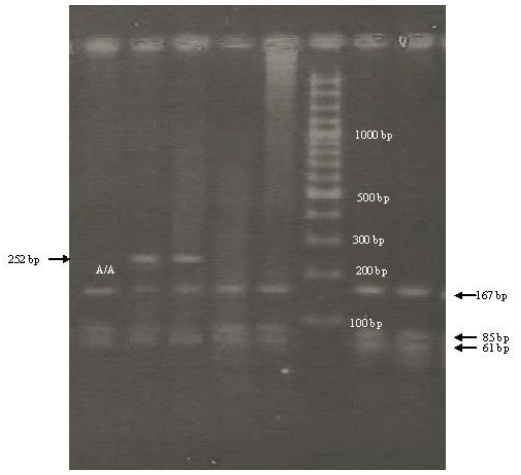

Figure 1. Genotyping of Ala98Val HNF-1α by *HeaIII* RFLP-PCR. Lines 1–5: The early-onset group ≤ 35; Line 6: Gene rulers™ 100 bp DNA ladder; Lines 7-8: The late-onset > 35 group. A/V: Alanine/ Valine genotype; A/A: Alanine/Alanine genotype.

No mutation was detected in all patients of late-onset > 35 group. There were no rare homozygotes (TT) for all participants. Of case diagnosed ≤ 35 years and control participants, 9.8% and 4% respectively were heterozygous (CT), giving an OR of 1 (95% CI: 0.13 to 11.42, p = 0.99). No significant change in T-allele frequency was detected in the early-onset diabetic patients (OR: 1.96, 95% CI: 0.27 to 22.06, p = 0.68). The mean ages of onset of diabetes and their mean BMI in participants with HNF-1α mutation were 32.5 ± 3.1 years and 27.4 ± 1.9 kg/m², respectively. Table 3 shows the clinical and biochemical characteristics of the studied participants. Mean fasting glucose, triglyceride, cholesterol, and LDL-C levels showed an increase in the early-onset and the late-onset groups compared with the control group (p < 0.02). The early-onset diabetic patients had lower mean serum HDL-C levels than the healthy control participants (p < 0.004). No significant differences were found between the early-onset ≤ 35 group and the late-onset > 35 group cases in all clinical features (except fasting glucose and HDL-C). To study whether the HNF-1α Ala98Val mutation had consequences on the phenotype, we compared clinical variables between the diabetic participants with and without the HNF-1α mutation. There were no significant differences in age, BMI, fasting glucose concentration, blood triglyceride, cholesterol, LDL-C, and HDL-C between these patients (Table 4).

Table 4. Clinical and biochemical features of the diabetic subjects with and without the *HNF-1α* Ala98Val Mutation

	Diabetic mutation Carriers	Diabetic subjects without mutation
Number (n)	4	96
Age (years)	49.3±7.5	52.77±10.59
BMI (kg/m^2)	27.4±1.9	26.43±2.99
FBS (mg/dL)	196.5±57.8	186.74±63.12
Triglycerides (mg/dL)	147.5±27.8	155.84±52.08
Cholesterol (mg/dL)	230.8±72.4	198.69±36.15
LDL-C (mg/dL)	150.2±45.6	128.33±36.52
HDL-C (mg/dL)	41.3±9.0	38.78±6.85

BMI, Body mass index; FBS, Fasting plasma glucose; LDL-C, Low density lipoprotein-cholesterol; HDL, High density lipoprotein-cholesterol; Data are presented as means ± SEM.

Discussion

We have identified Ala98Val mutation in the HNF-1α gene in four of 41 patients whose diabetes onset was before 35 years of age. No mutation was detected in patients whose diabetes onset was after 35 years of age. Although the prevalence of the HNF-1α Ala98Val mutation has been reported for many countries, the prevalence of the HNF-1α Ala98Val mutation in type 2 diabetes has not been previously reported in Iranian patients. In the present study, the HNF-1α Ala98Val mutation was found in 9.8% of the early-onset type 2 diabetes. Sahu et al. reported that the overall frequency of HNF-1α Ala98Val mutation was 14% in Indian patients with early-onset type 2 diabetes (Sahu et al., 2007). Anuradha et al. showed that in Asian Indians, the Ala98Val polymorphism of HNF-1α is associated with earlier age at onset of type 2 diabetes (Anuradha et al., 2005). Common variations in the HNF-1α gene have been associated with impaired insulin secretion (Chiu et al., 2003; Urhammer et al., 1997). The Ala98Val variant may influence transcriptional activity and insulin secretion in vivo, data from Caucasians suggests that the association is likely to only modestly increase the risk of adult T2DM (Holmkvist et al., 2006).

A previous study in Scandinavian participants has suggested an association of valine 98 allele with early-onset familial T2DM (Lehto et al., 1999). The SNPs rs1920792, rs1169288 (Ile27Leu), and rs1800574 (Ala98Val) showed nominal association with type 2 diabetes in the Scandinavian samples. However, these SNPs were not associated with type 2 diabetes in another sample of 4,400 individuals from North America and Poland (Weedon et al., 2005). A concurrent large association study in individuals from the UK indicated that common variation in HNF-1α is not associated with type 2 diabetes, with the exception of the Ala98Val polymorphism (3%) (Weedon et al., 2005; Winckler et al., 2005). In the present study, this allele was not associated with an early-onset of T2DM. This is the first study on the association of the HNF-1α Ala98Val polymorphism with early-onset of type 2 diabetes from Iran.

We did not find any association of GLP-1R Thr149Met polymorphism with type 2 diabetes. The GLP-1R is one of the key targets in the management of type 2 diabetes mellitus with actions including regulation of insulin biosynthesis and secretion (Drucker and Nauck, 2006). Several groups have examined normal and diabetic populations for natural mutations in the GLP-1R. The Thr149Met polymorphism was discovered in a diabetic patient with defective glucose-induced insulin secretion but was not found in non-diabetic control participants (Tokuyama et al., 2004).

Our data revealed that the HNF-1α Ala98Val and GLP-1R Thr149Met polymorphisms are not associated with an increased risk of type 2 diabetes in the studied population. Screening for the HNF-1α Ala98Val and GLP-1R Thr149Met mutations was performed only in the 100 type 2 diabetes individuals, and hence we cannot provide an exact frequency for these mutations in T2DM. The association with HNF-1α Ala98Val and GLP-1R Thr149Met polymorphisms needs to be confirmed in a larger cohort of Iranian patients with T2DM.

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Research Article

The significance of C-terminal NLS sequences of VirD2 in its nuclear localization in *Saccharomyces cerevisiae*

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Abstract

Agrobacterium tumefaciens is capable of gene transfer to both plant and non-plant organisms. Indeed, upon infection of eukaryotic cells, *Agrobacterium tumefaciens* transfers a piece of its tumor inducing (Ti)-plasmid, called T-DNA, to the host cell nucleus, which subsequently integrates into the host genome. The VirD2 virulence protein which has relaxase endonuclease activities covalently binds to the 5'end of T-DNA and facilitates its transfer, nuclear localization and integration into the host genome in collaboration with the interacting proteins of the host cell. The VirD2 is essential for *Agrobacterium*–mediated transformation of both plants and non-plant cells. Here, using yeast Green Flourescent Protein (yGFP) technology, we studied the subcellular localization of VirD2, expressed in the model eukaryote *Saccharomyces cerevisiae*. Fluorescence microscopy showed that an N-terminal yGFP fusion of VirD2 (i.e. 5' GFP-VirD2 3'), was located in the nucleus of yeast. With C-terminal fusions of VirD2 to yGFP (i.e. 5' VirD2-GFP 3'), no particular subcellular concentration of fluorescence was seen. This further confirms nuclear localization of VirD2 in eukaryotic cells and more importantly highlights the role of Nuclear Localization Signal sequences (NLS) of the C-terminal of VirD2 in this phenomenon.

***Keywords*:** VirD2, Nuclear delivery, GFP, *Agrobacterium, Saccharomyces cerevisiae*

Introduction

Nuclear gene delivery possesses great potential for its use in basic science, biotechnology, agriculture, and medicine. Developing gene transfer technologies has become one of the most intensively investigated strategies for current basic and clinical research. *Agrobacterium tumefaciens* is a Gram-negative phytopathogen which is able to transfer and integrate up to 150 kb single-stranded DNA (ssDNA) into the infected cell's cytoplasmic and nuclear genome (Soltani et al., 2008). This ability is mainly due to the presence of a tumor inducing (Ti) plasmid in *Agrobacterium*. The Ti plasmid encodes a number of virulence proteins (Vir) that mediate the formation of a single stranded DNA copy (T-strand) of a part of the Ti-plasmid and transferring of it across the kingdom barriers to integrate into the host genome (Citovsky et al., 2006). Upon induction of the virA-virG two-component regulatory system, the virulence (vir) regulon expresses several Vir effector proteins which play different roles in the tumor induction process. Among those, the VirD2 relaxase together with VirD1 and VirC1 is responsible for the formation of the T-strand. The VirD2 virulence protein covalently bounds to the liberated 5′ phosphate of the T- strand and through combined action of three NTP-binding/hydrolyzing proteins VirB4, VirB11, and VirD4 translocates, as a pilot protein, the T-strand to the recipient cell via a Type IV Secretion System. Inside the host cell around 600 VirE2 proteins cover the T-single strand. Both VirD2 and independently transferred nuclear localization signal sequence (NLS) containing VirE2 facilitate the import of the T-complex into the host nucleus. VirD2 has two nuclear localization sequences (NLS), one located in its N-terminal region and the second bipartite NLS sequence located in the C-terminal region (Wang et al., 1990; Howard et al., 1992). It has been shown that both VirD2 and VirE2 proteins have interaction with plant importn proteins implicating host factors in the nuclear entry of T-complex (Ballas and citovsky, 1997; Tzfira et al., 2001; Li et al., 2005).

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Furthermore, VirD2 interacts with a number of plant cyclophilins, a conserved cyclin-dependent kinase-activating kinase (Cak2M), and the TATA-binding protein (TBP) (Bako et al., 2003). Inside the host nucleus, VirD2 relaxase may influence the integration of T-DNA into the genome, although this is largely mediated by host factors (van Attikum et al., 2001; 2003). The unique mechanism by which *Agrobacterium* translocates any ssDNA molecule offers novel possibilities for gene transfer into fungal and mammalian cells (Soltani et al., 2008; Haghighi et al., 2013). Here, we studied the subcellular localization of the pilot protein VirD2 in yeast cell as a model eukaryote and the role of C- and N-terminal of NLS in this event.

Materials and Methods

Strains and media

E. coli strain XL1-blue (*supE44 hsdR17 recA1 endA1 gyrA46 thi relA1 lac−F' [proAB+ lacIq lacZΔM15 Tn10*] Tc^r) was used for all cloning processes (Stratagene). *E. coli* was grown at 37°C in Luria-Bertani (LB) or TB medium containing either 100µg/ml ampicillin or 60 µg/ml kanamycin. S. cerevisiae strain CEN.pk113-3B (*MATα his3Δ1 ura3-52*) was used for the green fluorescent protein (GFP) localization studies. All yeast strains were grown at 30°C in either YPD or MY supplemented with appropriate nutrients, i.e. 20 µg/ml adenine, 30µg/ml histidine, 20 µg/ml leucine, 30µg/ml lysine, and 20 µg/ml tryptophan (Sherman, 1991; Zonneveld, 1986).

Nucleic acid manipulations

All nucleic acid manipulations for plasmid construction were performed by standard protocols (Sambrook et al., 1989). For plasmid DNA isolation from *E. coli*, the QIAprep mini spin kit (Qiagen) was used. For isolation of plasmid from the yeast cells the same kit was used, after adding lyticase (1 mg/ml) to buffer P1. Isolated plasmids from yeast were amplified in *E. coli* XL1-blue.

Plasmid constructions

For the construction of pGBDKc1-virD2 the 3'-end of the virD2 open reading frame, lacking the first 379 bp, was obtained by PCR on plasmid pVD43 (Rossi et al, 1993). After digestion with *Sal*I and *Bgl*II, this part was cloned into pGBDK-C1 (van Hemert et al, 2003) digested with the same enzymes. The 5'-end of the virD2 open reading frame (379 bp) was obtained by PCR on plasmid pVD43 (Rossi et al, 1993) using the primers VirD2SalIp2 (5'-ACGCGTCGACGTCATGCCCCGATCGCGCTC AAG-3'), introducing a *Sal*I restriction site upstream of the ATG start codon, and VirD2p2 (5'-TATTCGGTCCTTCCTGTCTCTAGGTCCCCCC-3'). Subsequently, this fragment was digested with *Sal*I and introduced into the *Sal*I site of pGBDK containing the 3'-end of VirD2.

To make fusions between VirD2 and yeast enhanced GFP, an *Xma*I-*Eco*RI fragment with virD2 obtained from pGBDKc1-virD2 plasmid was cloned into the *Xma*I-*Eco*RI restriction sites of pUG34, pUG35 and pUG36 GFP-vectors (U. Güldener and J. H. Hegemann, unpublished data). In pUG34-virD2 and pUG36-virD2 vectors, virD2 is tagged with GFP at its N-terminus, and in pUG35-virD2 vector, virD2 is tagged with GFP at its C-terminus, and expressed under control of the *MET17* (*alias MET25*) promoter. Plasmids are listed in Table 1. New constructs were confirmed by both restriction analyses and DNA sequencing (BaseClear, The Netherlands). The GAD-fw primer 5'-GATGAGAAGATACCCCACC-3' was used for sequencing of the samples of the genomic libraries.

Table 1. Plasmids used in this study.

Plasmid	Features
pGBDKc1.*vir*D2 (pRUL1131)	*ADH1* promoter, Gal4 BD, AmpR, *TRP1*, Kan, ori, carrying *vir*D2
pUG34	*MET25* promoter, *HIS3*, CEN6/ARS4, AmpR, ori, N-terminal yGFP fusion site
pUG34-*vir*D2 (pRUL1146)	Expresses N-terminal yGFP fusion to VirD2
pUG35	*MET25* promoter, *URA3*, CEN6/ARS4, AmpR, ori, C-terminal yGFP fusion site
pUG35-*vir*D2 (pRUL1147)	Expresses C-terminal yGFP fusion to VirD2
pUG36	*MET25* promoter, *URA3*, CEN6/ARS4, AmpR, ori, N-terminal yGFP fusion site
pUG36-*vir*D2 (pRUL1148)	Expresses N-terminal yGFP fusion to VirD2

Transformation protocols

E. coli XL1-blue was transformed using regular heat shock protocol (Takahashi et al., 1992). For transformation of *S. cerevisiae* strains lithium acetate protocol was carried out (Gietz and Woods,

2002) and transformants were selected on MY medium supplemented with appropriate nutrients (Zonneveld, 1986).

Microscopy

For 4', 6-Diamidino-2-phenylindole (DAPI) staining of nuclei, overnight cultures from yeast strain CEN.pk113-3B containing GFP-fused VirD2 were harvested by centrifugation and resuspended in 1 ml of 70% ethanol (Hašek and Streiblová, 1996). After 5 min, the cells were again harvested and resuspended in 25 µL of 0.1 µg/ml DAPI. 5 µL of DAPI-stained yeast suspensions were then used for microscopy. Accordingly, 5 µL of overnight cultures were taken for fluorescence microscopy with a Zeiss Axio-plan-2 imaging microscope. GFP was excited at 488 nm, and emission was detected at 514-564 nm.

Results

Plasmid constructions

To confirm the virD2 insertion in GFP-containing plasmids, the restriction enzyme analyses were followed by sequencing of the constructs. *Sma*I, *Eco*RI, *Eco*RV restrictions resulted in the expected bands for each construct (Figure 1). The constructs (pUG34-virD2, pUG35-virD2, and pUG36-virD2) were checked further by sequencing (Data not shown).

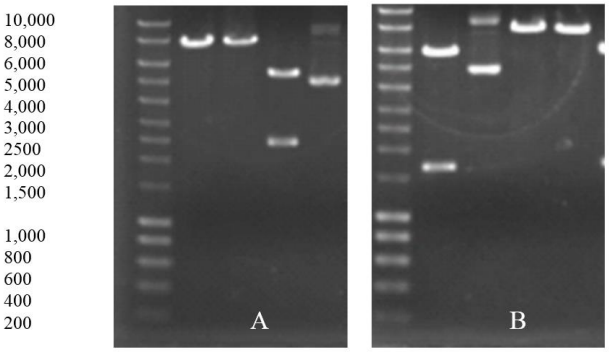

Figure 1. Representative restriction alanylses of virD2-gfp plasmid constructs. A, pUG35.virD2 restricted with EcorRI (ca. 7550 bp), SmaI (ca. 7550 bp), EcoRV (ca. 5132 bp & 2418 bp) & control respectively. B, pUG36.virD2 restricted with *Eco*RV (ca. 5852 bp & 1692 bp), control, *Eco*RI (ca. 7550 bp), *Sma*I (ca. 7550 bp), respectively. The numbers indicate the sizes of DNA ladder bands (bp).

To determine the localization of VirD2 in S. cerevisiae we expressed N- and C-terminal fusions of this protein with yGFP in this organism. Fluorescence microscopical analysis of yeast transformants was performed on overnight grown cells. Cells expressing an N-terminal fusion of VirD2 with yGFP (from both pUG34 and pUG36) revealed a typical nuclear localization of this protein (Figure. 1. A & D). DAPI staining of these cells confirmed the nuclear localization (Figure. 1. B & E). We were unable to detect nuclear localization of yGFP fluorescence in yeast cells expressing a C-terminal fusion of VirD2 with yGFP (Data not shown).

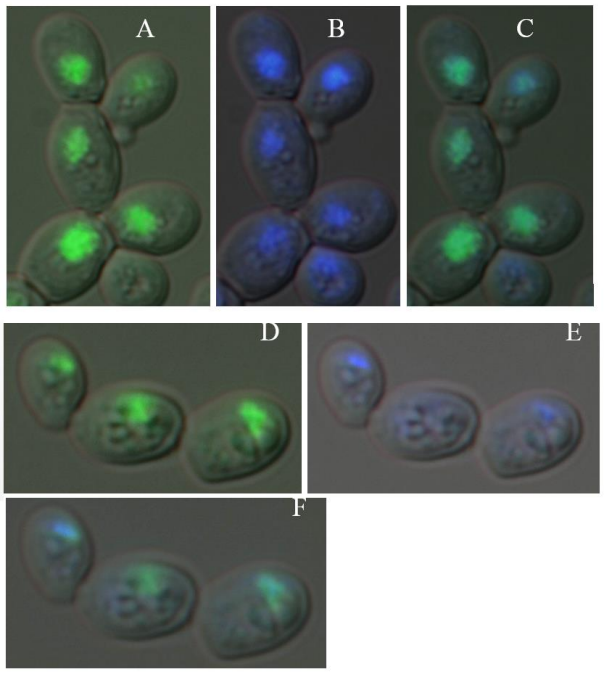

Figure 2. Subcellular localization of VirD2 fused at its N-terminus to C-terminus of GFP from both pUG34 (A-C) and pUG36 (D-F) in S. cerevisaie strain CEN.pk113-3B. A & D, fluorescence microscopy of expressed GFP proteins. B & E, DAPI staining of the same cells. C & F, Superimposition of figures A/B and D/E. Cells are visualized by a Zeiss Axio-plan-2 imaging microscope.

Discussion

Currently, gene transfer is limited largely by the fact that the issue of nucleic acid delivery has not been adequately resolved (Anderson, 1998). The nature's genetic engineer *Agrobacterium tumefaciens* translocates any ssDNA molecule to both eukaryotic and prokaryotic cells (Soltani et al., 2008). This offers novel possibilities for gene transfer into any eukaryotic cells. For a better understanding of the function of VirD2 in eukaryotic cells, the genetic model organism S. cerevisiae was recruited as a host to analyze the subcellular localization of VirD2 expressed in its cells.

The T- DNA of *Agrobacterium* transferred to the host cell needs to translocate to the nucleus to integrate in the genome. Inside the cytoplasm of

the host cell the T-DNA which is bound to VirD2 most likely is covered by VirE2 proteins. Both VirD2 and VirE2 have nuclear localization signals (NLS) sequences which mediate the import of the whole T-complex into the nucleus of plant cells (Citovsky et al., 1992; Tinland et al., 1992). We hypothesized that this might also happen in other eukaryotic cells as *S. cerevisiae*. C-terminal and N-terminal fusions of the virD2 to yGFP were expressed in yeast cells to visualize the subcellular localizations of them. As it is shown in Figure 1, VirD2 fused at its N-terminus to yGFP localized almost exclusively to the nuclei of *S. cerevisiae*. Nuclear localization of VirD2 in yeast cells is consistent with its localization in plant and mammalian cells (Citovsky et al., 1992; Tinland et al., 1992; Relić et al., 1998; Ziemienowicz et al., 1999; Ziemienowicz et al., 2001). However, with C-terminal fusions of VirD2 to GFP, fluorescence was largely uniform throughout the cells without nuclear concentration. VirD2 has an NLS sequence located in its N-terminal region and a bipartite NLS sequence located in the C-terminal region (Wang et al., 1990; Howard et al., 1992). In plants, it has been shown that N-terminal sequences of VirD2, containing 70% of the protein, could target β-galactosidase to the nucleus (Herrera-Estrella et al., 1990). It has also been shown that both the C- and N-terminal sequences of VirD2, when fused at their C-terminus to β-galactosidase, were able to direct β-galactosidase to the nuclei of plant cells (Tinland et al., 1992). Also, either the N- or C-terminus of VirD2 was sufficient to target the GFP fused protein to the nucleus of mammalian cells (Relić et al., 1998). In contrast, import of DNA into the nucleus of mammalian cells by VirD2 is dependent on the C-terminal NLS of VirD2 (Ziemienowicz et al., 1999; 2001). Similarly only the C-terminal NLS of VirD2, not the N-terminal NLS, fused to the C-terminus of β-glucoronidase targets the recombinant protein to the plant nuclei (Howard et al., 1992). The discrepancy between those and our observations could be due to the effect on NLS function of different reporter genes fused N- or C-terminally to VirD2, the different cells used for localization studies and different lengths of virD2 used. Our observation indicates the significance of C-terminal NLS in VirD2 localization in yeast nuclei. C-terminal fusions of VirD2 to GFP may block the function of the NLS, but when GFP is fused to the N-terminal region of VirD2 the NLS at the C-terminus is still functional and mediates the nuclear localization of the recombinant protein. Overall, in accordance to its function in plant and mammalian cells, VirD2 moves to the nucleus of its fungal host cells, and this function is mainly based on its bipartite NLS sequence located in the C-terminal region of the protein.

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Research Article

In silico analysis of chimeric recombinant immunogen against three diarrhea causing bacteria

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Abstract

Shigella and *Escherichia* belong to the *Enterobacteriaceae* family which are the cause for most of the diarrheal cases in the world. *Shigella* can cause bacterial dysenteries and shigellosis. One of the most effective proteins for pathogenesis is invasion plasmid antigen C (IpaC). Other bacteria like Enterotoxogenic (ETEC), Enterohemorrhagic (EHEC), and *E.coli* can also cause diarrhea and produce intestinal disorders. Colonization factor antigen I (CFA/I), a critical virulence protein for these infections, has two subunits i.e. CfaB and CfaE. EHEC Attachment of bacteria is the main step of infection with intimin playing the key role in this function. This study was designed to elicit protection against the majority of diarrheal pathogens via development of polyvalent vaccine against *Shigella*, ETEC and EHEC. *In silico* techniques are as best tools to design new vaccines. For this purpose the immunogenic epitopes of CfaB, IpaC and Intimin were identified through bioinformatic tools and were then selected as major antigens to construct a chimeric protein (CII). The humoral and cellular immunities were analyzed bioinformatically. Prediction of allergens and mapping of IgE epitopes were carried out. The bioinformatic analysis showed each domain was folded separately in fusion structure. CII had many T and B cell epitopes in both linear and three-dimensional structures. This prediction of the chimeric construct had the potential to induce CD4+ and CD8+ immune responses against these pathogens. In addition CII could be accessible to surveillance by the immune system in mouse and human. In conclusion, *in silico* analysis showed that this chimeric protein can be used as a vaccine against *Shigella*, ETEC and EHEC simultaneously.

***Keywords*:** Intimin; CfaB; IpaC; recombinant vaccine; chimeric protein

Introduction

Diarrheal diseases are among the most common causes of death in the world that can spread from person-to-person via the fecal-oral route. High rates of disease are found in daycare centers, hospitals and nursing homes. In addition, diarrhea is frequently reported in food-borne and water-borne outbreaks. *Escherichia coli*, *Shigella* and *Salmonella spp*. are the most important causing diarrhea in the world. The diseases attributed to *E. coli* include childhood and traveler's diarrhea (ETEC), bloody diarrhea and hemolytic uremic syndrome (HUS) (Bhatnagar et al., 1993). *Shigella* is a major source of bacterial diarrhea in developing countries. The most important diarrheogenic *Shigella* strains are *S. flexneri*, *S. sonnei*, *S. Boydii* and *S. dysenteriae* and untyped *Shigella* strains (Katouli et al., 1990). Diarrhea is the condition of having three or more loose or liquid bowel movements per day. The loss of fluids through diarrhea can cause dehydration and electrolyte imbalances. One of the most important bacteria producing severe diarrhea is *S. flexneri*, a gram-negative intracellular pathogen responsible for bacillary dysentery (shigellosis) (Terry et al., 2008b) which includes severe inflammation, fever, abdominal cramping, and ulceration of the colonic mucosa (Kueltzo et al., 2003; Nazarian et al., 2013). As estimated earlier, 164.7 million shigellosis occur per year, of which 1.1 million cases result in death, with a high rates of infant mortality in underdeveloped countries (Jennison and Verma, 2004). An initial important step in pathogenesis is the invasion of colonic epithelial cells that resulted in cytoskeletal rearrangements at the site of bacterial contact in the host cell.

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These localized changes lead to the formation of filopodia and trap the pathogen within a membrane-bound vacuole that is rapidly lysed, finally bacterium access the host cell cytoplasm (Picking et al., 2001). For the invasion of epithelial cell, *S. flexneri* requires encoding genes on a large virulence plasmid. The IpaA–D proteins play a central role in this process (Tran Van Nhieu et al., 1999). Upon host cell contact, IpaB and IpaC are embedded into the host cell membrane and generate a pore like transmembrane channel called the translocon. Effecter proteins can pass through the translocon into the host cell cytoplasm (Terry et al., 2008b; Tran Van Nhieu et al., 1999). Filopodial and lamellipodial extensions are induced when IpaC has access to the cell cytosol antibodies recognizing the C-terminal region of IpaC between residues 297 and 349. These data argue that the C-terminal domain of IpaC needs to interact with components present in the cell cytosol in order to promote actin polymerization (Tran Van Nhieu et al., 1999). The C-terminal tail residues 344-363 appears to contain a major effector function of IpaC required for cellular invasion (Terry et al., 2008b). In the vast majority of the cells, secretion of IpaC via the TTSS occurs at one pole of the *Shigella* cell during epithelial cell invasion. Destabilization of the cadherin complex by IpaC may modulate this transmission allowing bacterial enhanced multiplication within infected cells. Thus, IpaC might be a multifunctional protein that controls the invasion process in a finely tuned manner, and is, therefore, an important effector of *Shigella* pathogenesis (Barzu et al., 1997). (Picking et al., 2001).

Enterotoxigenic *Escherichia coli* (ETEC), is another important etiological agent of travelers' diarrhea and a main cause of infantile death in developing countries (Luiz et al., 2008, Nazarian et al., 2013). ETEC pathogenesis depends on the ability to produce the heat-stable (ST) and/or heat-labile (LT) enterotoxins after attachment of bacteria to the intestinal epithelia with colonization factors CFs or CFAs (Luiz et al., 2008). Colonization factor antigen I (CFA/I) fimbriae contains a polymer consisting of 1,000 copies of the major pilin subunit CfaB, and one or a few copies of the tip-residing adhesive minor subunit CfaE (Li et al., 2009). It thus seems that CfaB subunit is a carbohydrate-binding protein which especially interacts with a number of carbohydrate sequences present in human small intestinal glycosphingolipids and glycoproteins. Carbohydrate-binding activity donates the attachment of CFA/I-fimbriated *E. coli* to host intestinal epithelium. Indeed, monoclonal antibodies directed against this protein could inhibit the binding of CFA/I-expressing cells to human intestinal cell (Jansson et al., 2006). Therefore, CfaB may be important targets for vaccine development.

Enterohemorrhagic *Escherichia coli* (EHEC) O157:H7, a human pathogen that causes bloody diarrhea and hemolytic uremic syndrome leading to kidney failure and even death. Cattle are the main reservoirs of EHEC O157:H7 strains and the associated diseases are brought by ingestion of undercooked or raw milk (Dean-Nystrom et al., 1998). EHEC belongs to a family of pathogens with ability to produce attaching and effacing (A/E) lesions characterized by degeneration of the epithelial intestinal microvilli. (DeVinney et al., 1999). The first gene for A/E activity is eae encoding intimin, an outer membrane adhesion protein essential for intimate bacterial attachment to eukaryotic host cells. Intimin is a 94-kDa outer membrane protein in enterohemorrhagic Escherichia coli (EHEC) (Tran Van Nhieu et al., 1999). Intimin is required for colonization of E. coli O157:H7 and mediates bacterial attachment to the plasma membrane of infected cells. eae is a part of a chromosomal island called the locus of enterocyte effacement (LEE). The LEE encodes a type III secretion system (TTSS) which includes a translocated intimin receptor (Tir) and three secreted proteins EspA, EspB, and EspD. These proteins are required for signal transduction in mammalian host cells and A/E lesion formation (Amani et al., 2010; Vlisidou et al., 2006). The Tir-Intimin interaction triggers actin cytoskeletal rearrangements, resulting in pedestal formation (Amani et al., 2009). Intimate attachment to the host cell leading to the formation of A/E lesions is an essential feature of EHEC pathogenesis. The *eaeA* gene plays a key role in the pathogenesis of the AE lesion therefore intimin is a critical factor for invasion (Shaikh et al., 2003).

Studies on the different intimins have shown that receptor-binding activity is localized to the C-terminal 280 amino acids (Int280). An experimental vaccination with the carboxy-terminal of intimin induced strong response of specific antibodies in serum and colostrums of pregnant swines (Law, 2000). In this work a new structural model containing three effective pathogenic factors with antigenic determinants of IpaC, Intimin and CfaB was designed. This chimeric gene was fused together by hydrophobic linkers and codon optimization for expression in *E. coli* was carried out. The chimeric protein structure and its ability to induce CD4+ and CD8+ immune responses against

these pathogens were predicted by *in silico* approaches.

Materials and Methods

Antigenic segment selection

cfab, ipac and eae sequences were obtained from GenBank. In order to identify the general and conserved antigenic fragments in these bacterial strain sequences, multiple sequence alignments were performed by Clustal W software (EBI, UK) at (http://www.ebi.ac.uk/Tools/clustalw2/ Thomopson et al., 1994).

Bioinformatic analysis of chimeric protein

For optimization of chimeric gene expression in *E. coli*, the Genscript Optimization Gene TM algorithm (www.genescript.com, piscataway. newjersy USA) was used. The secondary mRNA structure and mRNA stability was predicted by GeneBee (http://www.genebee.msu.su/services /rna2_reduced.html), mfold Web Server (Zuker, 2003) (http://mfold.rna.albany.edu/?q=mfold/RNA Folding-Form) and RNAfold web server.

Secondary and tertiary structure prediction

The secondary protein structure for each selected segment and the complete designed construct was predicted by PSIPRED (http://bioinf.cs.ucl.ac.uk /psipred/), SSpro8 (http://scratch.proteomics.ics.uci.edu/index.html) and GOR IV (http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=npsa_gor4.html)(Garnie r et al., 1996).

Using ALPHAPRED (http://www.imtech.res.in /raghava/alphapred/index.html) (Wang et al., 2006) alpha turns residues were predicted in protein sequence. This method is based on the neural network training on PSI-BLAST generated position specific matrices and PSIPRED predicted secondary structure. Also different types of beta-turns such as Types I, II, IV, VIII were predicted by BetaTPred (http://www.imtech.res.in/raghava /betaturns/) (Kaur and Raghava, 2002).

For analyzing 2D structure stability, Ramachandran plot was drawn. For 3D protein structure predictions, several software based on homology modeling were used. They included SWISS-MODEL (http://swissmodel.expasy.org /workspace/)(Schwede et al., 2003), CPH models (http://www.cbs.dtu.dk/services/CPHmodels/) (Lund et al., 2002), phyre (http://www.sbg.bio.ic.ac.uk/~phyre/) (Kelley and Sternberg, 2009), FOLDpro and (PS)2 (http://ps2.life.nctu.edu.tw/) that combine PSI-BLAST, IMPALA, T-Coffee in both template selection and target-template alignment. The final three dimensional structures were built using the modeling package MODELLER (Eswar et al., 2007). Whole structures were then built by ab initio modeling using I-Tasser (http://zhanglab.ccmb.med.umich.edu/I-TASSER/) (Zhang, 2008). BHAGEERATH-H and Robetta software (http://robetta.bakerlab.org/index.html) \(Kim et al., 2004) combining homology and ab-intio modeling for Protein Tertiary Structure Prediction was used.

Prediction of immunogenic epitopes

B-cell epitopes: The linear B-cell sequences were obtained from ABCpred (Saha and Raghava, 2008) using Recurrent Neural Network, Bcepred (Saha and Raghava, 2004) base on using physico-chemical properties and BepiPred (http://www.cbs.dtu.dk/services/BepiPred/) (Ole and Morten, 2006) using a combination of a hidden Markov model and a propensity scale method. The residues were analyzed to predict discontinuous B cell epitopes using DiscoTope 1.2 Server (http://www.cbs.dtu.dk/services/DiscoTope/) (Haste Andersen et al., 2006).

MHC Binding Peptide

Prediction of MHC Class-I Binding Peptide was obtained from ProPred-I (http://www.imtech.res.in /raghava /propred1/) (Singh and Raghava, 2001) and nHLAPred (Zhang et al., 2009) and NetMHC (http://www.cbs.dtu.dk/services/NetMHC/) (Buus et al., 2003). NetMHC 3.2 server predicts binding of peptides to a number of different HLA alleles using weight matrices. Prediction of MHC Class-II Binding Peptide was performed by HLA-DR4Pred HLA-DR4Pred (Bhasin and Raghava, 2004b). The HLA-DR4Pred is a Support Vector Machine (SVM) and artificial neural networks (ANNs) based HLA-DRB1*0401(MHC class II alleles) binding peptides prediction method. But MHC molecules are highly polymorphic and MHC haplotype antigens of BALB/c mice are H-2Kd, H-2Dd, H-2Ld for MHC I and IAd, and I-Ed for MHC II. NetMHC 3.2 and ProPred-I was performed to predict H-2Kd, H-2Dd and H-2Ld.

The Rankpep (http://imed.med.ucm.es/Tools /rankpep.html) (Reche et al., 2002; Reche et al., 2004) is software for prediction of binding peptides to both Class I and Class II MHC molecules.

T-cell epitopes: CTLPred (http://www.imtech.res.in/raghava/ctlpred/) (Bhasin and Raghava, 2004a) is a direct method for prediction of CTL epitopes crucially related to MHC class I using Quantitative Matrix (QM), SVM and ANN in subunit vaccine design.

IgE epitopes: Prediction of allergens and mapping of IgE epitopes were obtained from AlgPred(http://www.imtech.res.in/raghava/algpred/) (Saha and Raghava, 2006). Algpred allows to predict allergen using SVMc + IgE epitope + ARPs (allergen-representative peptides) BLAST + MAST.

Protease and Hydropathicity prediction

Prediction of proteasome and immune-proteasome cleavage sites in CII obtained from Pcleavage (http://www.imtech.res.in/raghava /pcleavage/)(Bhasin and Raghava, 2005) using SVM and NetChop 3.1 Server (http://www.cbs.dtu.dk/services/NetChop/)(Saxov أ، et al., 2003) using ANN. Hydropathicity of CII was predicted by IEDB (http://tools.immuneepitope.org /tools/bcell/iedb_input)(Zhang et al., 2008) and ProtScale (http://web.expasy.org/protscale/) (Gasteiger et al., 2005).

Antigenic propensity and solvent accessibility

The probability of antigenicity of the construct was estimated by vaxijen (http://www.ddg-pharmfac.net/vaxijen/VaxiJen/VaxiJen.html)(Doytchinova and Flower, 2007). Antigenicity Prediction in single and assembled forms of selected segments was performed at http://www.pbcpeptide.com /Feedback.htm by Kolaskar & Tongaonkar Antigenicity and ANTIGENpro (http://scratch.proteomics.ics.uci.edu/). Prediction of solvent accessibility in CII was performed by Recombinant Protein Solubility Prediction (http://biotech.ou.edu/#r)(Davis et al., 1999; Wilkinson and Harrison, 1991), NetSurfP ver. 1.1, SARpred(Garg et al., 2005) using multiple sequence alignment and secondary structure and IEDB (http://tools.immuneepitope.org/tools/bcell /iedb_input).

Prediction of subcellular localization: Subcellular localization of CII in gram-negative bacteria was predicted by CELLO (http://e093.life.nctu.edu.tw /index.html) and PSLpred (http://www.imtech.res.in/raghava/) (Bhasin et al., 2005). PSLpred is a hybrid approach-based method integrating PSI-BLAST and three SVM modules.

Results

Antigenic segment selection

Entire cfaB protein (Bouzari et al., 2010, Nazarian et al., 2012), 282 amino acids from C-terminal of intimin (Amani et al.)and 64 amino acids in C-terminal of ipaC (Terry et al., 2008a) were selected. EAAAK (Arai et al., 2001) linker was used between each segment(Figure1).

CfaB	linker	Intimin 282	linker	IpaC 64

Figure1: Schematic model which shows the construction of whole of CfaB, Intimin 282 and Ipac 64, bound together by the linkers.

Bioinformatic analysis of chimeric protein

The CII mRNA structure (Figure 2) had a free energy of -361.3 Kcal/mol. The analysis of the sequence encoding the optimized chimeric gene and the wild type is shown in Figure3. As a result of reduction of overall GC content to 51.56%, the mRNA stability and ribosomal binding was optimized for transcription by changing the stem loop. The codon adaptation index (CAI) was increased. Restriction sites interfering with cloning, instability elements, and all the *cis*-acting sites were removed. The necessary restriction sites of *Eco*RI and *Hin*dIII were at the ends of the sequence for subsequent cloning.

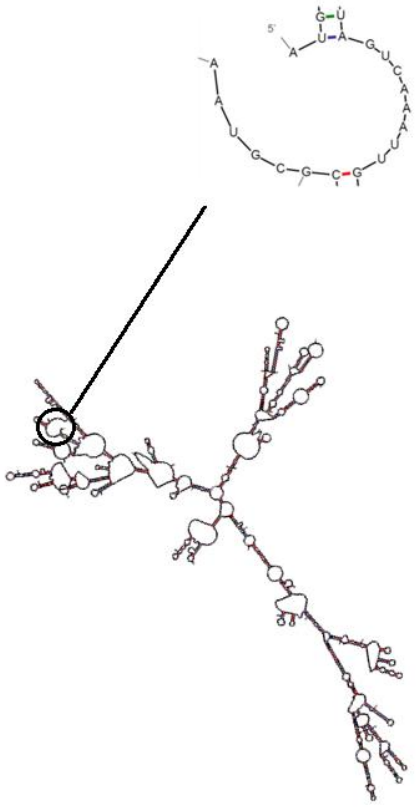


Figure 2: Analyzed of CII mRNA stability and first nucleotide position in this structure.

Secondary and tertiary structure prediction

The secondary structure, alpha and beta turn achieved by several online programs is given in Figure 4. No difference was seen comparing 2D structure of each selected segment and the complete designed construct. Secondary structure stability determined by Ramachandran plot is shown in

Figure 5. Tertiary structures predicted with several softwares are shown in Figure 6 and the linkers in this structure are highlighted in Figure7.

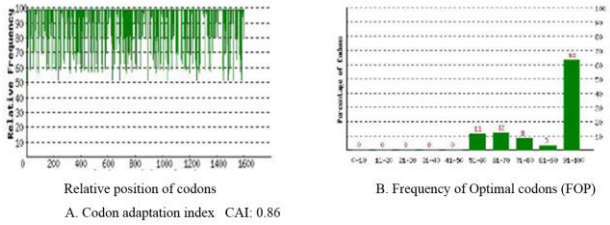


Figure 3: The sequence of the chimeric gene (CII) was optimized by changing some factors to increase gene expression. A) Codon adaptation index (CAI), B) Frequency of Optimal codons (FOP).

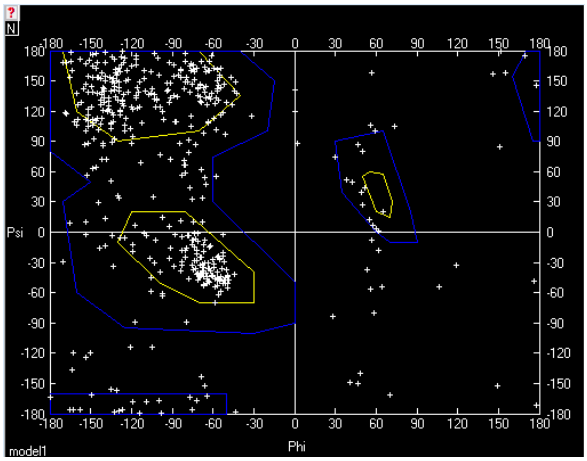


Figure 4: Evaluation of CII stability based on a Ramachandran plot.

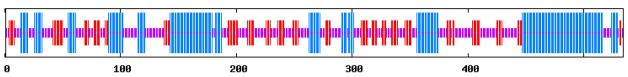


Figure 5: Analysis of CII protein secondary structure.

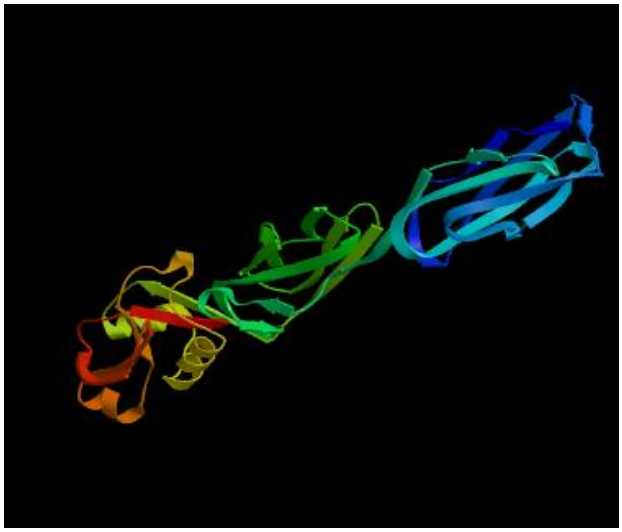


Figure 6: *Ab initio* modeling for prediction of the 3D structure of CII. The result was viewed by Rasmol software.

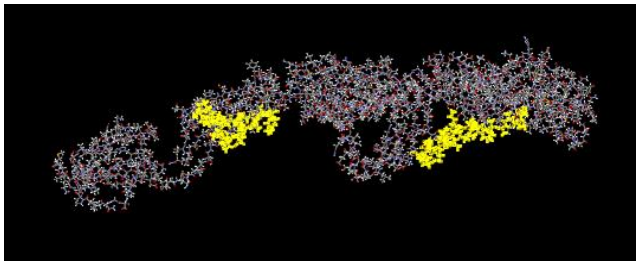


Figure 7: Analysis of the linkers' position in 3d structure of CII.

Prediction of immunogenic epitopes

B-cell epitopes: Bcepred utilized seven different physico-chemical scales including hydrophilicity, flexibility, mobility, accessibility, polarity, exposed surface, turns, and antigencity for prediction existing Continuous B-cell epitopes (Table 1). ABCpred is based on recurrent neural network, which ranked according to their score obtained and above the chosen threshold value (Table 2).

Table1: Epitope predicted CII chimeric protein by physico and chemical properties based on Bcepred

Prediction parameters	Epitope positions and segments
Hydrophilic	48-59, 110-117, 147-164, 180-186, 222-238, 242-250, 252-262, 282-288, 302-311, 321-329, 339-349, 412-424, 450-466, 482-505, 515-527.
Flexibility	32-41, 50-56, 107-116, 219-234, 237-246, 299-307, 327-334, 336-345, 409-423, 479-499, 513-525.
Accessibility	34-45, 48-59, 62-71, 92-98, 153-164, 167-175, 177-186, 189-197, 199-211, 221-236, 270-279, 296-302, 309-318, 339-351, 360-369, 394-403,411-421, 450-466, 472-509, 515-527.
Turns	49-56, 205-212.
Exposed surface	52-58, 271-279, 360-366, 412-418.
Polarity	39-45, 92-98, 153-164, 177-184, 269-279, 450-466, 472-482, 485-493.
Antigen propensity	56-63, 67-83, 114-121, 136-146, 194-200, 332-339, 372-380, 383-390, 433-439, 505-512.

Table 2: The predicted B cell epitopes by ABCpred obtained by trained recurrent neural network.

Rank	Sequence	Start position	Score
1	YNLITQNPLPGVNVNT	426	0.95
2	SYTIKAPSYMIKVDKQ	349	0.94
3	AWIKQTSSEQRSGVSS	409	0.93
4	DGTYSWYSENTSIATV	307	0.90
5	SKTFESYRVMTQVHTN	38	0.88
6	VKLADTPQLTDVLNST	61	0.87

Table 3: The result of prediction for MHCI antigens of BALB/c mice

MHC-Db	10-18, 24-32, 61-73, 110-118, 185-193, 199-213, 229-237, 264-280, 312-320, 350-358, 401-409, 420-428, 436-447, 471-480
MHC-Dd	18-30, 65-73, 85-94, 107-118, 137-145, 189-197, 203-212, 231-242, 273-312, 333-350, 353-361, 377-385, 420-428, 431-439, 466-474, 503-513
MHC-Kb	18-26, 39-47, 65-79, 99-107, 110-118, 134-142, 212-220, 232-241, 264-272, 293-301, 311-319, 362-373, 377-385, 396-407, 420-429, 440-448, 498-514
MHC-Kd	10-18, 24-41, 110-118, 135-146, 168-179, 186-194, 295-303, 311-320, 365-377, 397-408, 420-434, 472-480, 505-517
MHC-Kk	2-16, 52-61, 64-81, 97-105, 112-120, 148-166, 168-194, 231-239, 259-278, 284-292, 311-338, 351-359, 365-373, 380-388, 399-411, 415-429, 449-485, 499-517, 524-532.
MHC-Ld	10-21, 26-50, 66-74, 81-97, 104-112, 136-144, 205-221, 243-251, 291-301, 378-386, 390-398, 401-410, 414-422, 432-449, 481-489.

MHC Binding Peptide: Prediction for MHC I antigens of BALB/c mice is shown in Table 3. RANKPEP denoted binding peptides to Class II MHC molecules in mice (Table 4).

T-cell epitopes: T-cell epitope was directly predicted by CTLpred (Table 5).

IgE epitopes: Algpred predicted allergens based on similarity of known epitopes with any region of CII. The protein sequence does not contain experimentally proven IgE epitope.

Table 4: Prediction of MHC II antigens in mice by RANKPEP

I-Ek	407-415, 194-202, 187-195, 462-470, 457-465, 452,460, 160-168, 155-163, 150-158, 359-36
I-Ab	169-177, 125-133, 246-254, 365-373, 434-442, 104-112
I-Ad	483-491, 88-96, 403-411, 146-154, 466-474, 482-490, 246-254, 445-453, 208-216
I-Ed	87-95
I-Ag7	189-197, 391-399, 448-456, 458-466, 453-461, 156-164, 151-159, 131-139, 464-472

Table 5: Prediction of T-cell epitope by CTL pred

Peptide Rank	Start Position	Sequence	Score	Prediction
1	360	KVDKQAYYA	0.990	Epitope
2	439	VNTPNVYAV	0.990	Epitope
3	524	TASQIAGNI	0.990	Epitope

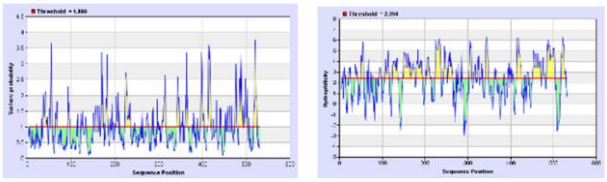
Table 6: Predicted peptide with high accessibility by Emini Surface Accessibility Prediction

No.	Start Position	End Position	Peptide	Peptide Length
1	35	46	SPASKTFESYRV	12
2	92	97	TTAKEF	6
3	168	173	KFDQTK	6
4	179	184	IKADKT	6
5	203	208	NGQPVN	6
6	223	235	GKSQTQATTGNDG	13
7	272	277	LKIDNK	6
8	308	316	GTYSWYSEN	9
9	341	348	TSGDKQTV	8
10	361	366	VDKQAY	6
11	395	403	ANKYSHYSS	9
12	412	420	KQTSSEQRS	9
13	484	492	ASSKQAEEA	9
14	494	503	QVSKEASQAT	10
15	517	524	INQSKNST	8

Antigenic propensity and solvent accessibility

For building a construct with higher antigencity, different states of chimer was checked. This model

had the highest score (0.7086) in vaxijen. CII has a 79.2 percent chance of insolubility when over expressed in *E. coli*. The average of surface accessibility was 1.00 and the maximum and minimum of CII surface accessibility were 3.763 and 0.081 respectively. Surface accessibility graph was predicted by Emini Surface Accessibility Prediction (Figure 8A). Table 6 shows the predicted peptides with high accessibility.



A B

Figure 8: Analizing of CII surface accessibility(A) and hydrophibisity(B) by IEDB

Protease and Hydropathicity prediction

Pcleavage did not find the residues with proteasome cleavage site in antigenic sequence. Hydropathicity graph was drawn by Parker Hydrophilicity Prediction (Figure 8B). The minimum and maximum Hydropathicity ranked between -3.043 and 6.326 with an average rank of 2.394.

Discussion

An effective vaccine against ETEC in adult travelers is licensed in few countries, and the development of a new ETEC vaccine is sought (Svennerholm and Tobias, 2008). In dysentery caused by *Shigella*, treatment with appropriate antibiotics shortens the duration of the symptoms and reduces the duration of host excretion of the pathogen. Sulfonamides, ampicillin, and nalidixic acid were used to be the first-line therapies. Gradually, *Shigella* became resistant to each of these antibiotics. Ciprofloxacin is the first choice of antibiotics recommended by the World Health Organization for treating shigellosis and currently, increasing resistance to ciprofloxacin has been documented (Ahs et al., 2010). Antibiotic use in a case of EHEC O157:H7 may place the patient at greater risk of HUS (Dundas et al., 2001) and could increase the amount of toxin produced by strains of Shiga toxigenic group of *Escherichia coli* (STEC) bacteria (Kimmitt et al., 2000). The abilities of Shigella to enter epithelial cells require Ipa proteins which play prominent roles in the infection process (Picking et al., 2001). Intimin is encoded by the *E. coli* attaching and effacing (eae) gene required for intimate adhesion to epithelial cells and cytoskeletal reorganization (Tran Van Nhieu et al., 1999). CfaB is a major subunit in CFA/I fimbriae polymer of ETEC bacteria (Jaumouille et al., 2008) (Terry et al., 2008b). CfaB is an important target to prevent ETEC invasion. In this work we designed a construct containing epitopic segments of CfaB, Intimin and IpaC. A subunit vaccine containing these epitopes could be an effective way of simultaneous protection aganist shigella, EHEC and ETEC. Antigen index of different orientations of the epitopes in the chimeric protein was analyzed. The bacterial threshold of 0.4 renders this protein as a suitable antigen. The structure shown in Figure 1 was the most optimum choice. The codon usage of chimeric gene adapted to the codon usage of highly expressed genes in *E. coli*. GC content, CpG dinucleotides content, Cryptic splicing sites, Repeat sequences, Premature PolyA sites, Negative CpG islands, Internal chi sites and ribosomal binding sites, RNA instability motif and Restriction sites that may interfere with cloning were considered. In the mRNA structure, the position of start codon stands in the loop (Figure 2) that helps better ribosome binding and subsequent start of translation. Because of low ΔG (-361.3 Kcal/mol.), the mRNA is expected to be highly stable. This phenomenon is further supported by Ramachandran plot. When the secondary structure of chimeric protein was compared with 2D structure of single proteins, no significant changes were noted. Each domain in 3D protein structure maintained their conformations in fusion structure. The presence of helix-forming peptide linker caused reduced interference between the fragments. Finally localization prediction in gram negative bacteria shows CII as a cytoplasmic protein.

For immunological study, the humoral and cellular immunity was analyzed. CII had many B cell epitopes in both linear and three-dimensional structure. B cell response is critical, especially in production of IgA and IgG. This prediction of the chimeric construct had the potential to induce CD4+ and CD8+immune responses against these pathogens in the BALB/c model.

The CII peptide fragments are carried to the surface of the presenting cell on MHC proteins, which present the fragments to helper T lymphocytes. This stimulates B cells to make antibodies, macrophages to destroy any intracellular pathogen multiplying and cytotoxic T cells to kill infected target cells. The prediction of allergenic proteins is becoming very important due to use of modified proteins in therapeutics. This prediction study also revealed that the chimeric construct was not allergen. In conclusion, as this chimeric structure has a potential to induce humoral and cellular immunity response, CII could be a candidate

subunit vaccine against EHEC, ETEC and shigella.

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Research Article

Allelic polymorphism of *K-casein*, *β-Lactoglobulin* and *leptin* genes and their association with milk production traits in Iranian Holstein cattle

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Abstract

The purpose of this study was to investigate the polymorphism of *K-casein* (*K-CN*), *β-Lactoglobulin (B-LG*) and *leptin* (*LP*) genes in Iranian Holstein cattle by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) technique. DNA was extracted from blood samples of 139 cows using a modified phenol chloroform method. Association between *K-CN*, *B-LG* and *LP* genes' polymorphism with milk production traits were investigated using mixed procedure of SAS software. The frequencies of AA, AB and BB genotypes for *K-CN* (0.72, 0.18 and 0.10), *B-LG* (0.43, 0.28 and 0.29) and *LP* (0.24, 0.63 and 0.13) were also calculated. Statistical results revealed a significant association between AA and BB genotypes of the *K-CN* gene with milk production and milk protein percentage, respectively. Also, BB genotype of the *B-LG* gene and AA genotype of the *LP* gene showed a significant association with protein percentage and milk production ($P<0.05$), respectively. Therefore, it is feasible to improve milk production traits in these herds using the studied genes.

***Keywords*:** Holstein cattle, *K-casein, β-Lactoglobulin, leptin* gene, PCR-RFLP, milk production traits

Introduction

Genotypes that affect milk production traits could be used as genetic markers in marker-assisted selection programs. Although genes with large effects only show part of the genetic differences between animals, identifying these genes could be a great help to estimate more accurate breeding values in breeding programs (Hayes et al., 2009). Many studies have been conducted on milk protein polymorphisms to determine frequencies of genetic variants and their associations with milk production traits (Erhardt, 1996; Vohra et al., 2006). Milk proteins in ruminants are divided to two main groups named caseins and whey proteins. *B-LG* is the main part of the whey proteins in ruminant species, and its locus is located on chromosome 11 (Simpson et al., 1998). This gene is very polymorphic and has about 12 alleles (Godovac-Zimmermann et al., 1996). The most frequent ones are A and B variants that are the results of point mutations in exon 4 of the *B-LG* gene leading to two amino acid changes at positions 64 (Asp→Gly) and 118 (Val→Ile) (Rachagani et al., 2006). It has been shown that high protein content in ruminant's milk with AA genotype is due to more *B-LG* protein in their milk (Ng-kwai-hang, 1998; Glantz, 2011). *K-CN* is a polymorphic gene in cattle that has 5 exons and 4 introns. This gene has several forms and 13 alleles have been reported for this gene so far (Prinzenberg et al., 2008). The most common alleles are A and B. A is different from B because of substitution of two amino acids at positions 136 (Thr → Ile) and 148 (Asp → Ala) (Grosclaude et al., 1972). The effect of A and B forms on milk traits in cattle have been widely studied and most of the results suggest a positive effect of BB genotype compared with AA genotype on milk traits such as fat and milk protein (Lunden et al., 1997).

Leptin is a hormone that is involved in the regulation of food intake, long-term energy balance, body weight, reproductive and immune system. Leptin is encoded by the OB gene that has three exons and is located on chromosome 4. Recently, polymorphisms have been reported for this gene suggesting that it as a candidate affecting the milk production and its components (Buchanan et al., 2002; Buchanan et al., 2003). Due to the fact that nearly all of the dairy cattle in the industrial dairy farms in Iran are Holstein, this study aimed at investigating *K-CN*, *B-LG* and *LP* polymorphisms in Iranian Holstein cattle and their associations with milk production and components.

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Materials and Methods

Blood samples were collected from 139 Holstein cows belonging to six different dairy farms participating in the recording system of National Animal Breeding Center. DNA was extracted from whole blood using modified phenol chloroform method. In order to determine the quantity and quality of the extracted DNA, both spectrophotometric and gel monitoring methods were used. Optimization of PCR conditions were mainly done on three important factors including concentration of $MgCl_2$, PCR primer binding temperature and PCR temperature program. PCR reactions containing 50 ng of genomic DNA, 200 mM of each dNTPs, 25 mM of each primer, 2.5 mM $MgCl_2$, and 1 unit of Taq DNA polymerase enzyme, were carried out in a final volume of 15 µl. The 247 bp fragment, comprising a part of the IV exon and intron of the genomic DNA was amplified by using primers as suggested by Strazalkowska et al. (2002). A 422 bp fragment of intron 2 in bovine leptin gene was amplified by PCR using forward and reverse primers according to Liefers et al., (2002) and the primers used for amplification of κ-casein gene fragment were reported by Mitra et al., in 1998. The primers used for amplification are given in Table 1.

For amplification of *K-CN* gene, an initial denaturation step at 95°C for 4 min followed by 35 cycles of denaturation at 94℃ at 30 seconds, annealing at 58℃ at 30 seconds and extension at 72°C for 45 sec, and a final extension of 72°C for 5 min were set. PCR amplification programs for the *B-LG* and *LP* genes were similar to those of the *K-CN*, but the reactions were carried out at 60℃ as annealing temperature and synthesis step for *B-LG* was set at 70℃. PCR reactions were carried out using CG1-96 thermocycler (Corbet). The PCR products were visualized using electrophoresis on 2% agarose gel in 0.5X TBE buffer then 5 µl of *B-LG* and *K-CN* PCR products were digested overnight at 37°C with 3 units of Hinf1 and HaeIII enzymes overnight at 37°C, respectively. The *LP* PCR products were digested with 3 units of *Kpn*2I restriction enzyme at 55℃ overnight. The digested products were separated on 3.5% agarose gel and detected with ethidium bromide staining. Allele scoring and counting were performed by comparing with the standard markers.

In order to perform association analysis, 2532 test day records, including production of milk protein, milk fat and milk somatic cell counts (SCC), collected during 2001-2006 from six Iranian Holstein herds were used. Determination of milk composition (fat, protein and somatic cell counts) was done using Ultrasonic Milk Analyzers (EKOMILK, Bulgarian). After Kolmogorov-Smirnov test on the data and insurance of the normality of the data, traits were included in the statistical model. Population genetic parameters such as allele and genotype frequencies, expected heterozygosity (H_E) and observed homozygosity (H_0) were calculated using PopGen 2.32 software. Likelihood ratio test ($G2_T$) was used to determine the Hardy-Weinberg equilibrium status of the loci (Guo and Thompson, 1992). In order to calculate the effect of different genotypes on milk production traits, a mixed model in accordance with repeated measurements was considered. Statistical analysis was performed based on Mixed Proc using SAS software (SAS software, 2002). The following model was used to analyze the association between milk yield, fat, protein and somatic cells counts with studied genotypes:

$$Y_{ijklmno} = \mu + P_i + S_j + H_k + T_l + G_m + R_{ijklm} + e_{ijklmno}$$

where $Y_{ijklmno}$= milk production, milk fat, milk protein or somatic cell score, µ= overall mean of the trait under analysis, P_i= effect of lactation with 6 classes (cows with 6 and more lactations were merged in one class), S_j= the calving season effect with four classes, H_k= fixed effect of herd with 6 classes, T_l= fixed effects of year with 5 classes, G_m= fixed effect of studied genes, $R_{ijklm=}$ the random permanent cow effect with mean 0 and variance S^2_{δ}, and finally $e_{ijklmno}$ is total residual effects with mean 0 and variance σ^2.

Table 1. Primers used for PCR amplification of of the experiment.

Locus	Sequence
K-CN locus	Forward Primer: 5′- ATC ATT TAT GGC CAT TCC ACC AAA G-3′ Reverse Primer: 5′- GGC CAT TTC GCC TTC TCT GTA ACA GA-3′
B-LG locus	Forward Primer: 5′- TGT GCT GGA CAC CGA CTA CAA AAAG-3′ Reverse Primer: 5′- GCT CCC GGT ATA TGA CCA CCC TCT-3′
LP locus	Forward Primer: 5′-ATG CGC TGT GGA CCC CTG TAT C-3′ Reverse Primer: 5′-TGG TGT CAT CCT GGA CCT TCC-3′

Results

The specific primers for the *K-CN* gene amplified a fragment corresponding to the exon 4 and intron 4 with a size of 350 bp. digesting this fragment by *Hinf1* enzyme produced three different

patterns. The first pattern produced two bands (226 and 84 bp), the second pattern produced three bands (134, 132 and 84) while the third pattern produced four different bands (226, 134, 132 and 84 bp) (Fig. 1). The AB genotype showed all four possible bands. The allelic frequencies reported in the studies for *K-CN* was similar to Lundén *et al*. (1997) and Cardak (2005).

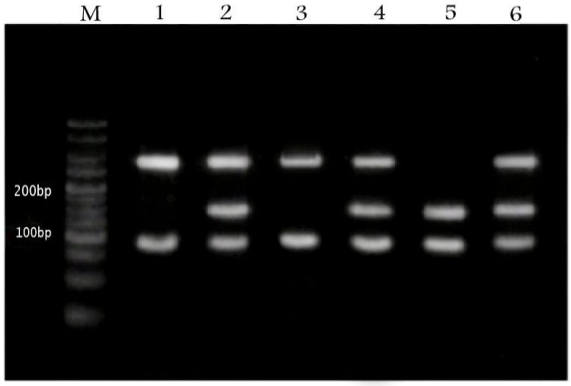

Figure 1. The gel electrophoresis pattern of the fragments produced from PCR on *K-CN* genes and restriction digested with *HinfI* enzyme. Lane 2, 4 and 6, represents AB, lane 1 and 3 represent BB and lane 5 represent AA genotypes and M is a 50 bp ladder marker.

The specific *B-LG* primers amplified a 247 bp fragment corresponding to its exon 4. Digestion of this fragment by *Hae*III showed three patterns. In the first pattern, two bands of 148 and 99 bp (AA genotype), in the second pattern two bands of 99 and 74 bp (BB genotype) and in the third pattern three bands of 148, 99 and 74 bp (AB genotype) were observed (Fig. 2). The allelic frequency for *B-LG* that reported by Tsiaras *et al*. (2005) and Hill (1993), confirm the frequencies obtained in current study.

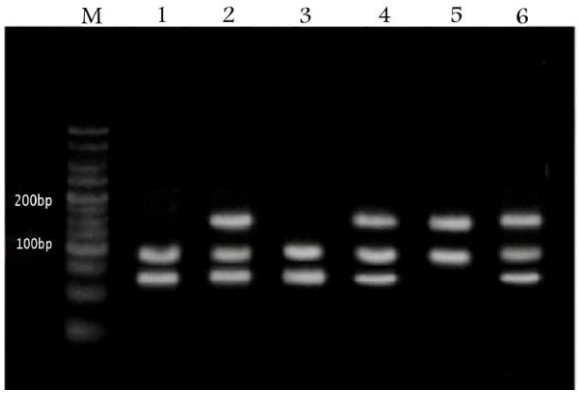

Figure 2. Pattern of *B-LG* gene after treatment with *HaeIII* enzyme. Lane 1 and 3 represent BB, lane 2, 4 and 6 represent AB and lane 5 represent AA and M is a 50 bp ladder marker.

The specific *LP* primers amplified a 94 bp fragment of its exon 2. Digestion of this fragment by *Kpn*2I showed three patterns. In the first pattern, a 94 bp band (AA genotype), in the second pattern two bands with the size of 19 and 74 bp (BB genotype) while in the third pattern three bands with the size of 19, 74 and 94 bp (AB genotype) were observed (Fig. 3). Similar studies by Moravčíková and et al. (2012) on Slovakian cattle and Buchanan *et al*. (2003) on Angus and Herford cattle, revealed that the predominant allele was A. In both populations, they observed all three genotypes AA, AB and BB, as we did in current study.

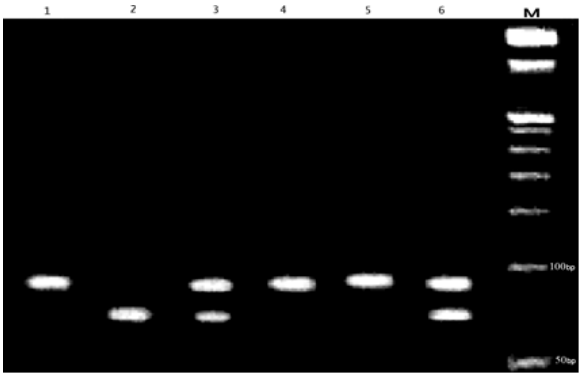

Figure 3. The gel electrophoresis pattern of fragments produced from PCR on *LP* gene after treatment with *Kpn2I* enzyme. Lane 1, 4 and 5 represent AA, lanes 3 and 6 represent AB and lane 2 represent BB and M is a 50 bp ladder marker.

Allele frequency obtained in the present study was in consistence with the previous reports. However, in some cases, may be for the reasons like different statistical designs, i.e type of continuous variables (for example, breeding values, average deviation of daughter performance or production records), different calving stage and environmental conditions, this consistency was not observed.

Table 2. Gene and genotype frequencies for K-casein, β-Lactoglobulin and leptin genes in Iranian Holstein cattle determined by PCR-RFLP.

Loci	Gene frequency		Genotype frequency			H_E^*
	A	B	BB	AB	AA	
K-CN	0.81	0.19	0.1	0.18	0.72	0.31
B-LG	0.57	0.43	0.29	0.28	0.43	0.49
LP	0.61	0.39	0.13	0.63	0.24	

* H_E: Expected Hetrozygosity

The gene and genotype frequencies for *K-CN*, *B-LG* and *LP* loci and the expected heterozygosity are summarized in table 2. Based on the results of likelihood ratio test, all of the loci were not in Hardy-Weinberg equilibrium (P<0.05). This might be due to the deviation of the population from the Hardy-Weinberg equilibrium conditions such as existence of selection, genetic drift, small size of the population, etc. The allele frequencies observed in this study is fully consistent with previous reports (Buchanan *et al.*, 2003; Prinzenberg *et al.*, 1996; Sharifzadeh *et al.*, 2012; Soria *et al.*, 2003).

Table 3. Statistical analysis and probability values of the tested loci

Trait	*K-CN*	B-Lactoglobulin	*LP*
milk production	0.040*	0.215	0.030*
fat percentage	0.414	0.594	0.122
protein percentage	0.075*	0.007*	0.113
somatic cell score	0.613	0.022	0.546

* Statistically significant at the probability level (P < 0.05).

Table 4. Comparison of the least square means and standard errors of milk production traits of different genotypes.

Genotype	milk production	milk protein %	milk fat %	milk somatic cell score
		K-CN		
AA	29.08±7.36[a]	3.09±0.27[b]	3.44±0.53	3.31±1.05
AB	28.11±5.89[b]	3.19±0.13[b]	3.36±0.29	3.331±0.68
BB	28.03±6.17[b]	3.20±0.36[a]	3.39±0.31	3.27±0.73
		B-Lactoglobulin		
AA	25.58±5.79	3.11±0.38[b]	3.40±0.59	3.35±0.92
AB	28.76±8.08	3.16±0.19[b]	3.39±0.43	3.26±1.11
BB	28.74±7.34	3.27±0.27[a]	3.46±0.39	3.27±0.89
		LP		
AA	29.74±52.13[a]	3.15±0.18	3.20±0.39	3.32±0.5
AB	24.12±52.33[b]	3.16±0.10	3.25±0.23	3.36±1.00
BB	23.91±38.66[b]	3.17±0.12	3.23±0.39	3.37±0.90

LS means with different letters (a, and b) are significantly different (P < 0.05).

Undoubtedly, imported sperms from countries such as America, Canada, Australia and some European countries and their use in Iranian cattle industry affected this consistency. Tables 3 and 4 summarize the results of statistical analysis and least square means for different genotypes and traits. Results from analysis of variance, indicated significant herd and lactation effects on fat percentage, milk production and milk somatic cell (P<0.05). Also, *K-CN* locus had a significant relationship with milk production (P<0.05) and protein percentage (P<0.07). Comparison of the least square means showed significant difference between AA and BB genotypes of the *K-CN* gene for milk production and milk protein percentage. However, polymorphisms of this locus were not associated with milk fat percentage and milk somatic cell score. These results were confirmed by Tsiaras et al. (2005) that reported B variant of κ-CN gene has a favorable effect on protein yield. There are also some studies that failed to find any relationships between κ-CN genotypes with production traits (Hamza et al., 2011).

The BB genotype of the *B-LG* gene has shown a strong relationship with protein percentage (P<0.01) while the *B-LG* gene had no association with milk production, fat percentage and somatic cell score. It has also been shown that high protein content in ruminant's milk, with AA genotype, is due to more *B-LG* protein in their milk (Ng-kwai-hang, 1998; Glantz, 2011). In contrast of current results, several authors have reported no significant associations of genotypes on milk yield (Tsiaras et al., 2005). However, reports exist where β-LG genotypes have been positively associated with milk yield (Cardak, 2005; Heidari, 2009). The AA genotype of β-LG has also been shown to have a favorable effect on protein yield (Cardak, 2005; Bovenhuis *et al*., 1992). Also, there are only a few studies in literature trying to find an association between the polymorphism of κ-CN and β-LG with milk somatic cell count records. As one of the rare studies on this subject, Lunden et al., (1997) reported no association between the polymorphism of κ-CN and β-LG with milk SCC. This observation is in coincidence with the result of the current study.

Discussion

The results shows a significant association between AA genotypes of the *LP* gene milk production (P<0.05). Also, the same result was observed by Moravčíková and et al., (2012) in pinzgau cows with AA genotype on milk and protein yield.

Undoubtfully, the distribution of Holstein semen worldwide and using of imported semen extensively in the country farms has resulted in the complete accordance with the outcomes of the forementioned investigations. Possible divergence from Hardy-Weinberg expectations at each locus was evaluated. Both loci showed significant deviation ($p<0.05$) from Hardy-Weinberg proportions. These results were expected, because a number of basic underlying assumptions of Hardy-Weinberg equilibrium such as random mating, infinite population size and equal parental contribution in a breeding population were violated. In conclusion, the findings of this study indicated the existence of polymorphism in all of the three loci responsible for milk yield and compositions. These findings are in consistent with some of the previous studies. The relative consistency can be due to the global distribution of sperm produced in the Holstein breed around the world. Incorporating of the identified direct markers, linked to milk yield and composition to the breeding program for Holstein cattle in Iran can lead to a more rapid genetic progress for milk yield and composition.

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Research Article

Assessment of genetic stability of olive *in vitro* propagated by RAPD marker

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Abstract

The effect of sub culturing frequencies and different cytokinins (benzyl amino purine (BAP), 2-isopentenyl adenine (2ip) on genetic stability of micropropagated shoots from olive plant were investigated by using physiological traits and Random Amplified Polymorphic DNA (RAPD) markers. Axillary buds of the olive (cv. Dezful) plants were cultured and subcultured in DKW medium, supplemented with BAP (4 mgl^{-1}) or 2-ip (4 mgl^{-1}). In different hormone media and subcultures there were not significant differences in shoot proliferation rate. To amplify DNA, 18 arbitrary decamer primers were screened, out of which 16 primers generated clear and reproducible bands. All RAPD profiles from the micropropagated plants were monomorphic. The treatments included four successive subcultures in two hormone treatments (2ip or BAP) (4 mgl^{-1}) The 16 primers produced a total of 213 (an average of 13.31 band per primer) scorable bands. The dendrogram constructed on the basis of jaccard's similarity matrix, followed by UPGMA based clustering analysis showed that micropropagated plants were genetically stable and similar to the mother plant.

***Keywords*:** Micropropagation, genetic stability, *Olea europaea* L., RAPD

Introduction

Olive (*Olea europaea L.*) is one of most important fruit tree with a great commercial value that cultivated in many countries such as Iran and Mediterranean countries (Rugini, 1984). Considering the development of olive cultivation programs and difficulties that an experienced in olive vegetative proliferation, propagation of plants through in vitro culture is considered to be the most effective method. In vitro culture techniques provide an alternative means of plants propagation and a tool for crop improvement, rapid clonal propagation and obtaining high number of elite genotypes and conservation germplasm of endangered species (Vasil, 1998).

Micropropagation has been applied to the olive since 1980s (Zacchini and De Agazio,2004; Mendoza-de Gyves et al., 2008; Peyvandi et al., 2009a, 2009b; Ansar et al., 2009; Haq et al., 2009).

In this study, proliferation of shoots through the stimulation of axillary buds has been achieved by different cytokinins. Cytokinin influenced the shoot elongation by their effect on cell division and cell expansion. Growth regulators such as cytokinin may lead to generated genetic instability in tissue culture, namely somaclonal variation (Larkin and Scowcroft, 1981).

Among the micropropagation methods, propagation by axillary bud simulation is considered to bear low-risks of genetic instability (Rugini and Pesce, 2006; Peyvandi et al., 2009 a, 2009b) and widely used in micro propagation systems.

In commercial propagation programs, the most crucial concern is to retain the genetic and physiological characteristic of the mother plants and it is compulsory to check regularly the clonal fidelity or genetic uniformity of micro propagated plantlets (khawale et al., 2006). The genetic variation rate depends on the genotype, the explant source, the number of subcultures and duration of the culture period and the composition of the culture medium (Smith, 1998). Assessment of the genetic stability of *in vitro* derived clones is an essential step in the application of biotechnology for micro propagation of true to type clones (Diaz et al., 2003).

Phenotypic identification, based on a description of the morphological and physiological traits, can be used but some changes induced by *in vitro* culture cannot be easily observed because the structural difference in the gene product does not

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always alter its biological activity enough to be noticeable in the phenotype (Jin et al., 2008). When this occurs, somaclonal variations can be detected using morphological, cytological, biochemical and molecular methods (Al- Zahim et. al., 1999; Zhao et.al., 2005; Joshi and Dhawan, 2007) among micropropagated plants in many Taxa. Molecular techniques, particularly Random Amplified Polymorphic DNA (RAPD) have been proposed to be appropriate powerful tools for identification of somaclonal variation and establish genetic stability (Rahman and Rajora, 2001; Bennic et al., 2004; Javanmardi et al., 2011).

In the present study, the influence of different hormones and the number of subculturing on genetic stability in the micropropagated olive plants, using RAPD markers, is evaluated.

Materials and Methods

Plant material and micropropagation

Young shoots of an Iranian olive cultivar 'cv. Dezful' (10-15 cm long) were collected from a five-year old plant grown greenhouse. Leaves were excised and sterilized with commercial bleach (20%) for 5 minutes then rinsed in sterile distilled water (3 times). Apical buds of sterile shoots were removed and shoots were cut into single nod segments. Uninodal explants were cultured in DKW medium (Driver and Kuniyuki Walnut, 1984) with two different hormone treatments (2ip 4 mg/l)or (BAP4 mg/l). The carbohydrate source in tissue culture medium was mannitol (30 g/l). pH was adjusted to 5.7-5.8 (prior to autoclaving) and subsequently autoclaved for 20 min at 120ºC.

To study the effect of the number of subculturing and different hormone treatments on genetic stability of the micropropagated olive plants, sterile *in vitro* shoots were used for plant tissue culture. Two nodal explants of sterile shoots were subcultured for 4 times with 45 days interval in the same/ different tissue culture medium (DKW) including 2ip or BAP. All samples were kept in a growth chamber with 16h light/8 h dark photoperiod and 24 ± 2 ºC. After each subculture, the number of nods and branches raised from each explant and number of leaves and the length of the internods were measured for further analyses.

Experiments followed a randomized complete block design. 4 explants per jar and 8 replications per treatment were tested. Analysis of variance was performed by General Linear Model procedure (SPSS ver.14) and differences among the treatments were evaluated by Duncan Test ($p \leq 0.05$).

Molecular analysis

DNA extraction and PCR amplification

Three explants were randomly collected from cultures, each of four successive subcultures, and from the parental genotype cv. Dezful. DNA was extracted from the leaves of these explants using the CTAB (Cetyl–Trimethyl–Ammonium Bromide) method, described by Murry and Thompson (1980) with modification by De la Rosa et al. (2002), using approximately 1 gr of fresh tissue from each explant was powdered.

DNA was qualified by electrophoresis (3 V/cm) in 0.8% agarose gels (w/v). The DNA was visualized by ethidium bromide staining, and the original DNA solutions were then diluted to 10 ng/L for PCR reactions.

PCR amplification for RAPD analysis was carried out in a total volume of 20 µl, containing 20–40 ng template DNA, 1x PCR buffer "complete"(670 mM Tris-HCL, pH 8.8, 160 mM (NH4)2SO4, 0.1% Tween-20, 25 mM MgCl2), 200 µM dNTPs, 1 µm 10-mer primers (Operon Technologies Alameda California) and 0.5 unit of Taq polymerase . The amplification reaction was performed in a (Touchgene Gradient) thermocycler. After an intial denaturation at 94 ºC for 5 minutes, followed by 35 cycles consisting of a denaturation step at 94 ºC for 1 min, annealing step at 35 ºC for 1 min, extension step at 72 ºC for 2 min and a final extension at 72 ºC for 10 min. The PCR amplified products were separated by electrophoresis on 2% agarose gels and stained with ethidium bromide. The representative samples from each culture and sub-culture, for obtaining more band resolution, were separated by electrophoresis on 6% polyacrylamide gels (Sambrook et al., 2001).

A DNA ladder (Mix, Gene RulerTM, Fermentase) was used as molecular weight marker and the gels were stained with silver staining (Sambrook et al., 2001).

Data Analysis

Only consistently reproducible, well-resolved fragments, in the size range of 200 bp to 2500 bp were scored from 3 repeats. The presence or absence of the RAPD markers in the micropropagated plantlets obtained from each culture and subculture stages as well as in the mother cultivar trees in different treatments were evaluated. Bands of equal molecular weight and mobility generated by the same primer were considered to be identical.

RAPD reproducible fragments were scored as 1 for presence and 0 for the absence of DNA band in each sample. Genetic similarities between samples

were measured by the Jaccard's and Simple Matching similarity coefficient and the similarity matrix obtained was used to construct dendrogram using the UPGMA (Unweighted Paired Group with Arithmetic Average).

Table 1. The mean number of branch nodes, leaves and length of internodes in culture, first, second, third and fourth subcultured shoots. Different letter columns indicate significant differences ($p \leq 0.05$).

Step of Culture	Hormone (4 mgl^{-1}) 2ip	Hormone (4 mgl^{-1}) BAP	Branch	Node number	Leaves number	Length of internode (cm)
Culture	+	-	1.76_{a}	4.13_{a}	8.75_{ab}	10.36_{ab}
	-	+	1.79_{a}	4.13_{a}	9.91_{a}	11.74_{a}
First Subculture	+	-	1.58_{a}	2.27_{bcde}	4.08_{cde}	5.96_{de}
	-	+	1.52_{a}	2.00_{bcde}	4.03_{cde}	5.82_{de}
Second Subculture	+	-	1.51_{a}	3.33_{abc}	6.03_{bc}	7.22_{bcd}
	-	+	1.62_{a}	2.95_{abcd}	5.52_{bcd}	6.48_{de}
Third Subculture	+	-	1.74_{a}	3.21_{ab}	5.97_{bcd}	5.71_{de}
	-	+	1.53_{a}	3.38_{ab}	6.31_{bc}	7.30_{bcd}
Forth Subculture	+	-	1.53_{a}	3.27_{abc}	6.00_{bcd}	6.82_{de}
	-	+	1.39_{b}	3.27_{abc}	6.2_{bc}	876_{bcd}

Table 2. Total number and size range of the amplified fragments generated by 16 random decamer primers in axillary shoots of olive plants (cv. Dezful).

Sr.#	Primer name	Primer sequence (5′-3′)	No. of amplified fragment	Size range (bp)
1	OPC-01	TTCGAGCCAG	11	580-2200
2	OPC-02	GTGAGGCGTC	12	330-1550
3	OPC-03	GGGGGTCTTT	8	780-2500
4	OPC-04	CCGCATCTAC	11	440-2500
5	OPC-05	GATGACCGCC	10	470-1750
6	OPC-06	GAACGGACTC	14	450-2600
7	OPC-08	TGGACCGGTG	20	310-2400
8	OPC-10	TGTCTGGGTG	17	450-2500
9	OPC-11	AAAGCTGCGG	13	370-2100
10	OPC-12	TGTCATCCCC	16	470-2500
11	OPC-13	AAGCCTCGTC	11	485-2400
12	OPC-14	TGCGTGCTTG	13	360-1860
13	OPC-16	CACACTCCAG	12	440-2650
14	OPC-17	TTCCCCCCAG	11	480-2300
15	OPC-18	TGAGTGGGTG	18	430-2400
16	OPC-20	ACTTCGCCAC	16	360-1645
Total			213	

Results

Micropropagation

The explants on DKW medium with different combinations of growth regulators started to sprout and elongate after 45 days of culturing. After this period the growth rate of the subcultured explants at different conditions were recorded. Analysis of variance (ANOVA) performed for comparing the effects of different growth factors in successive subcultures indicated that there was no significant difference ($p<0.05$) between the effects of these two hormones on the number of nodes, branches and the leaves in the regenerated plants (Table 1). In both hormone treatments, it was observed that the growth factors in the culture step were more than the other steps of subcultures.

RAPD analysis

Three sets of PCRs were carried out for RAPD fingerprinting of each sample. 16 out of the 18 arbitrary initially screened decamer primers, produced clear and scored bands. A total of 3195 bands (number of samples analyzed × number of scorable bands in all tested primers) were generated, which exhibited homogenous banding patterns with the RAPD markers.

All of the mentioned fragments were monomorphic, confirming the genetic stability of the micropropagated plants. The 16 primers used in this analysis yielded 213 scorable bands with an average of 13.31 bands per primer. The size of amplified products ranged from 200 bp to 2500 bp. Among the used primers OPC-08 produced the highest number, 20, of bands while primer OPC-03 produced the lowest number, 8, of bands (Table 2). The size of the monomorphic DNA fragments produced by these two primers ranged from 310 bp to 2400 bp and 780bp to 2500bp respectively. As an example, the pattern obtained for primers OPC-04 and OPC-20 are shown in Figures 1 and 2. Genetic similarities between the micropropagated plants and the mother plant were scored by comparing their RAPD profile for each primer and calculating the coefficient of genetic similarity.

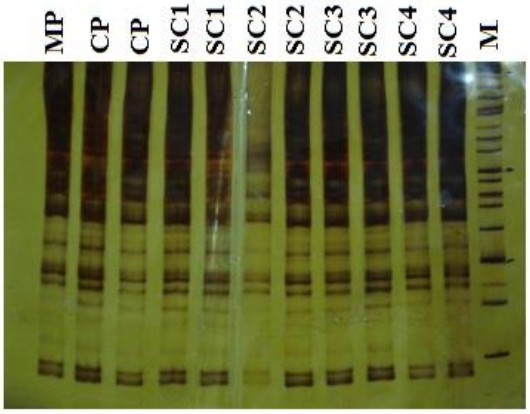

Figure 1. RAPD profile of micropropagated plants of *Olea europaea* L. generated by the primer OPC04 in the mother plant (MP), cultured (CP), and the subcultured microshoots in 2ip treatments. The abbreviations on top of each lane represent as, M: DNA ladder Mix, MP: the mother plant, SC1: the first subcultured plant, SC2: the second subcultured plant SC3: the third subcultured

plant, SC4: the forth subcultured plant; 2ip: 2-isopentenyl adenine, BAP: 6- Benzyl amino purine.

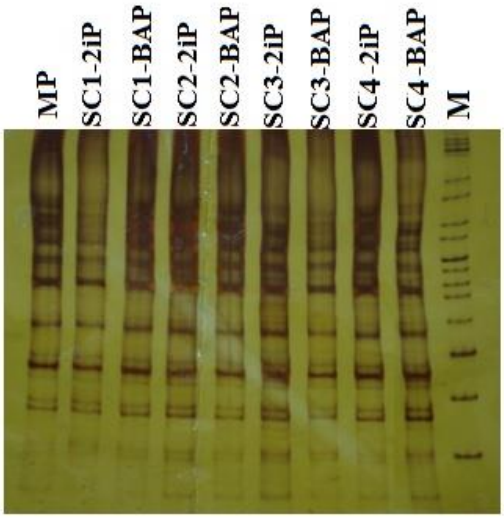

Figure 2. RAPD profile of micropropagated plants of *Olea europaea L.* generated by the primer OPC20 in the mother plant and subcultured microshoots. The abbreviations on top of each lane represent as, M: DNA ladder Mix, MP: the mother plant, SC1: the first subcultured plant, SC2: the second subculture plant SC3: the third subcultured plant, SC4: the forth subcultured plant; 2ip: 2- isopentenyl adenine, BAP: 6- Benzyl amino purine.

The dendrogram was constructed on the basis of jaccard's similarity matrix, followed by UPGMA (unweighted pair group mean average) based clustering analysis, which showed that the genotypes were grouped into single (in one major) cluster with the mother plant. The results indicate that plant regenerated in two different hormone treatments and in four successive subcultures had 100% similar to the mother plant.

Discussion

An important problem associated with plant propagation is the genetic stability among sub-clones derived from the original parents. The genetic integrity of the micrropropagated plants may lead to changes either in the phenotype or the genotype that can be determined with by different techniques. These variations are often undesirable, but still heritable (Brieman et al., 1987).

RAPD technique has been used to amplify regions of the genome of the plants regenerated from cultured cells, tissues or organs and useful for determination of genetic stability in the plantlets such as Ginger (Rout et al., 1998); Almond (Martins et al., 2004); Grape (Alizadeh and Kumar Singh, 2009); Date Palm (Kumaret al., 2010), Pistacia (Ehsanpour and Arab, 2009).

Of the 18 primers tested, 16 produced amplification products that were all monomorphic across the mother plant and plantlets (micro propagated plants). Similarity index value did not show distance between the parental and micropropagated plants even after increasing the number of subcultures at different hormone treatments. The results of the present study indicates that the genetic similarity between the parental plant and the *in vitro* propagated shoots from the subcultures in different hormone treatment were 100%.

Somaclonal variation in different plants have been widely studied (Rugini and Pesce, 2006). The presence or absence of variation depends very much on the source of explant and method of regeneration or on the source (callus, protoplast and cell) from them the shoots are regenerated (Larkin and Scowcroft, 1981). Micropropagation through axillary buds/organized meristems is generally considered to be a low risk method for genetic instability (Pierik, 1991; Martins et al., 2004) because the organized meristems are generally more resistant to genetic changes as compared to the unorganized calli under *in vitro* conditions (Shenoy and Vasil, 1992).

Present results showed no significant different ($p<0.05$) in physiological growth rate such as number of nodes, leaves, and branches as well as length of the shoots at all stages by different treatments. There are contrary reports on effects of two different hormonal treatment of BA and 2ip. While on olive, cultivar Kalamon (Dimassi-Theriou, 1994) showed that BA alone is more effective than 2ip and, Peyvandi (2009a) found that on olive cultivar Rowghani the 2ip was better than BAP. However our results here showed that there was not significant difference between application of two hormones (BAP,2ip).

Our finding are in agreement with Leva et al. (2002, 2009) who reported micropropagation did not affect morphological and genetic fidelity of tissue culture-derived olive plants. Lopes et al. (2009), using SSR method, reported no mutation between the donor trees and somatic embryos in two olive species. Also Brito et al. (2010) reported the genetic stability of two micropropagated wild olive species using flow cytometry and microsatellite markers.

According to our results, the number of subculturing and different hormonal treatments could not be affecting parameters to impose genetic instability. Also, the present study confirms the suitability of the micropropagation procedure in term of genetic stability and that the RAPD technique is sensitive enough for the somaclonal

variation analysis in the regenerated olive plants.

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Commentary

Developments toward an Ideal Skin Substitute: A Commentary

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Abstract

Skin grafting always has been considered a challenging task for the researchers and tissue engineers from its first introduction in 1871 by Reverdin. Skin substitutes, composed of degradable synthetic or biological components, are being considered as emergency replacements/grafts to the damaged skin. A number of technical developments in this filed have led to development of several skin substitutes, such as Biobrane®, Integra®, OrCel®, Suprathel® etc which are available for clinical utilization. From these, some characteristics, including infection resistance, water loss prevention, long shelf life, easy to store are set as criteria for assessment of the products. Post grafting problems associated with available skin substitutes questioned their reliability and reject them as an ideal skin substitute. Innovative tissue engineering approaches based on biological scaffolds and clinical grade stem cells could be an attractive alternative for available skin substitutes.

***Keywords*:** Tissue engineering, allografts, xenografts, epidermis, keratinocyte cultures, Skin Substitutes

Introduction

Skin is the largest protective organ of the human body, making up to 15% of the body weight. It acts as a functional barrier against the invasion of germs, body fluid loss, etc. (Lai-Cheong and McGrath, 2013). Skin is composed of three basic layers of epidermis, dermis and hypodermis. Epidermis is the outermost layer which is mainly composed of proliferating and non-proliferating keratinocytes (Arda et al., 2014). Accidental damaging of the skin, cutaneous wounds and burnings result in the severe and life threatening complications to the patients (Blais et al., 2013). Immediate replacement of the skin remained a clinical practice since the 19th century in the form of epithelial cell grafts (Reverdin, 1871). The limited amount of epithelial cells and donor sites are the major challenges in advantageous skin grafts. Conceptual approaches in the development of an ideal skin substitute for immediate replacement of damaged or wounded skin have remained as clinical interests for researchers, globally (Boyce, 2001; Balasubramani et al., 2001). Investigations in this area have resulted in introduction of the first skin substitute in 1981 by Burke and his colleagues (Burke et al., 1981). To date, a number of biological and synthetic skin substitutes are commercially available i.e. Biobrane®, Integra®, OrCel®, Suprathel® etc. Synthetic components are mostly organic polymers which are degradable and provide a regenerative environment for tissue regeneration. Biological skin substitutes are cellular products containing proliferative keratinocytes (Whitaker et al., 2008; Heimbach et al., 1988; Eisenberg and Llewelyn, 1998; Uhlig et al., 2007). Combinatory approaches using skin substitutes and dermal components i.e. fibroblasts have been applied for better wound healing (Eisenberg et al., 1998; Still et al., 2003; Veen et al., 2010). Better understanding of cellular and molecular mechanisms in skin regeneration is needed for the development of an ideal skin substitute (Bielefeld et al., 2013).

Classification of Skin Substitutes

Several skin substitutes are currently available for a variety of clinical applications. They can be classified into different categories, based on different criteria (Atiyeh et al., 2005; Horch et al., 2005). Almost all commercially available skin substitutes have been classified under the following three main headings (Ferreira et al., 2011).

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1. The first category has been classified based on their origin from skin layers. It is subdivided into epidermal (ESS), dermal (DSS) and dermal-epidermal skin substitutes (DESS). Orcel®, and Apligaft® are examples of this group (Hensen et al., 2001). ESSmembers, for example Epidex®, are derived from epidermal, keratinocytes, components of the skin, whereas DSS members are derived from the dermal components like fibroblasts and mesenchymal stem cells. OASIS wound matrix® is an example of the dermal skin substitutes (Ortega-Zilic et al., 2010; Hafner et al., 2006; Demling et al., 2004). Epidermal-Dermal composites are the third category of these skin substitutes to make them more effective for clinical purposes. OrCel® is an example of the epidermal-dermal skin substitutes (Veen et al., 2010).

2. Durability is the second factor in the classification of skin commercially available substitutes. These are further divided into temporary (TSS) and permanent skin substitutes (PSS). Temporary skin substitutes (TSS) provide transient physiologic wound closure, physical barrier to bacteria and creation of a suitable wound environment (Sheridan et al., 2001). Here are some currently available Temporary skin substitutes: Opsite®, Hydrofilm®, and Tegaderm® (Halim et al., 2010; Fikry and Bittner, 2013). Permanent skin substitutes (PSS) cover the wound permanently and replace the skin components in order to provide a more competent skin substitute than the thin autogolous skin grafts, e.g. Suprathel® (Uhlig et al., 2007).

3. Compatibility of the skin substitute is also an important factor in the classification of skin substitutes. Considering this, skin substitutes are classified into biological (BSS), synthetic (SSS) and bio-synthetic skin substitutes (BSSS) as shown in figure 1 (Ferreira et al., 2011). Biological skin substitutes, which act temporarily as replacement to skin, have the advantages of being relatively abundant in supply and not expensive. The biological skin substitutes have a more intact and native ECM structure which may allow the construction of a more natural new dermis. Having a basement membrane also allows excellent re-epithelialization. However, natural constructs can exhibit problems with slow vascularization of the material. The most widely used biological substitutes worldwide are cadaveric skin allograft, porcine skin xenograft, and CellSrpay® (Halim et al., 2010; Gerlach et al., 2011). Synthetic skin substitutes are constructed from non-biological molecules and polymers which are not present in normal skin (Veen et al., 2010). Due to their structures, these substitutes have their own advantages and disadvantages. For example the artificial composition and properties of these products can be much more precisely controlled. Various additives such as growth factors and matrix components can be added to facilitate the wound healing process. However, these synthetic skin substitutes generally lack basement membrane and their architecture does not resemble the native skin. Amongst the synthetic skin substitutes, available in the market, are Biobrane®, Dermagraft®, Integra®, Apligraft®, Matriderm®, Hyalomatrix® and Renoskin® (Halim et al., 2010; Demling, 1985). Biosynthetic materials are a combination of synthetic components with biological derived elements. Hyalomatrix® is the most favorable example of Biosynthetic skin substitutes (Myers et al., 2007).

Composition and Clinical Applications

EpiDex® is an example of epidermal skin substitute composed of autologous hair follicles. In technology, keratinocytes are grown in 1 cm-discs, with a silicone membrane, which are then grafted

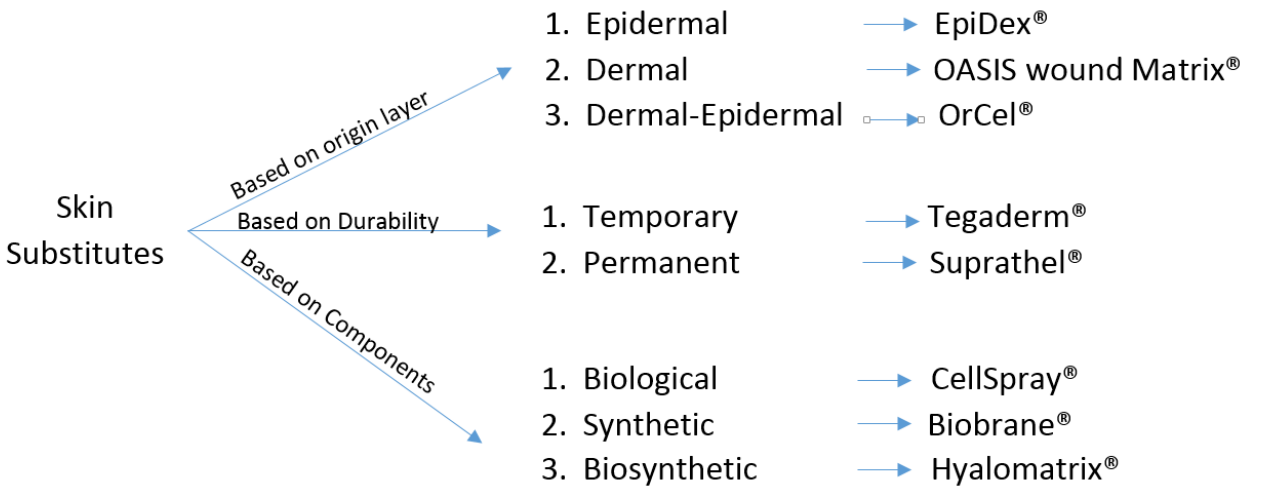

Figure 1: Classification of Skin Substitutes (S.S.)

onto the wound site (Ortega-Zilic et al., 2010; Hafner et al., 2006).

OASIS Wound Matrix® is an acellular dermal regeneration matrix, derived from swine jejunum submucosa (Demling et al., 2004; Brown-Estris et al., 2002), which leaves a structure composed of glycosaminoglycans, fibronectin, proteoglycans, and growth factors. It is commonly used in lower limb wound treatment and can be stored at room temperature (Niezgoda et al., 2005).

OrCel® is a bilayered cellular matrix which consist normal human allogeneic skin cells (epidermal keratinocytes and dermal fibroblasts) within a type I bovine collagen sponge. It is used in the treatment of chronic wounds and skin graft donor sites (Eisenberg et al., 1998; Still et al., 2003; Veen et al., 2010).

Tegaderm® is a temporary skin substitute for a temporary and small wound covering which is composed of Chlorhexidine Gluconate (CHG) IV gel (Fikry and Bittner, 2013). It has been proposed as an alternative approach to deliver autologous cells for chronic wounds (Chua et al., 2008).

Suprathel® is a purely synthetic skin substitute, composed of co-polymers of Lacto-capromer and polylactic acid (Uhlig et al., 2007). Clinically, it has been applied for the superficial partial-thickness burn wounds. As a synthetic substitute, it acts just as a protective barrier against the microbial invasion and relied on the patient's cells to improve their regeneration (Rahmanian-Schwarz et al., 2011).

CellSpray® is a cultured epithelial autograft suspension containing Ringer lactate solution, introduced in 1995 which is applied to the deep wound and stimulate the cells to regenerate the surface area (Gerlach et al., 2011). Its dependence on the culturing of autologous basal keratinocytes is a major challenge in clinical applications as an emergency skin replacement.

Biobrane® is a synthetic skin substitute containing an inner layer of nylon mesh and an outer layer of silastic. In clinics, it is applied on clean superficial burns (Demling, 1985).

Hyalomatrix® is a scaffold based bilayer skin substitute containing hyaluronan with autologous fibroblast and an outer silicone membrane which may limit colonization of cells in wound bed when applied clinically (Myers et al., 2007).

Conclusion and Perspectives

Cutaneous biology from the early use of its first autologous cell grafts, is experiencing a number of challenges for covering or replacement of injured or damaged skin. In spite of detailed understanding of physiological process in skin regeneration, researchers are still facing problems in the development of an ideal skin substitute. Stem cell based therapeutic and tissue engineering approaches are also gaining good deal of attentions. Reliable and xenobiotic-free keratinocyte culture techniques, (MacNeil, 2007), better understanding of the molecular mechanisms in the regulation of epidermal stem cells (Irfan-Maqsood, 2013), 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 (Shah et al., 1989; O'Kane and Ferguson, 1997; Ferguson and O'Kane, 2004) 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 remodeling still remained the major issues in skin bioengineering (Sriwiriyanont et al., 2013; Haifei et al., 2014). Associated problems with chemical scaffolds, perceives the ideas of biological membranes as alternatives (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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Letter to Editor

Therapeutic potential of Stem Cell Preconditioning for Ischemic Heart Diseases / Letter to the Editor

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Abstract

Preconditioning (PC), is an approach to improve therapeutic potential of stem cells against ischemic environment. PC has several advantages over other therapeutic techniques as this results in increase of transplanted stem cells recruitment, retention, survival and subsequently the induction of a more supportive environment within the damaged tissue via secretion of angiogenic factors. Special attention is needed to recognize new materials, compounds, and conditions to assess the feasibility of PC for being applied in clinics to treat the ischemic diseases.

***Keywords*:** stem cell preconditioning, cardiovascular diseases, ischemic heart diseases, progenitor cells

Introduction

Cardiovascular diseases especially ischemic heart disease are the most common cause of death and hospital admissions in most developing countries including Iran. From the perspectives of regenerative medicine, inducing neoangiogenesis and neovascularization in ischemic areas can be very helpful to restore organ perfusion and reduce the impacts of this problem. Various other therapies for cardiovascular diseases are clinically investigated and performed.

Recently, cell-based regenerative medicine using several cell sources and novel strategies e.g. preconditioning etc. have appeared as an alternative therapy for curing cardiovascular diseases (Wilschut et al., 2012). Preconditioning (PC), which is a well-known protective phenomenon activated by ischemic stress, where cells can sense and adapt to the environment by changing their cellular phenotypes and functions, has recently been applied in clinics to treat ischemic diseases (Yu et al., 2013). Different chemokine factors in preconditioned stem cells and ischemic heart and their role in angiogenesis, neovascularization, and regeneration of damaged tissues have been investigated. SDF-1/CXCR4 has been described as a vital chemokine axis in ischemic heart to orchestrate the rapid revascularization of injured and ischemic tissues. SDF-1 released by ischemic tissues, promotes the sprouting of small endothelial tubes from pre-existing capillaries, the egress of stem cells from bone marrow (BM), and their homing and differentiation into the injured tissue (Yu et al., 2013). PC strategies can affect the SDF-1/CXCR4 functions using different kinds of cells as shown in Table 1.

Table 1: Effects of some PC strategies on SDF-1/CXCR4 axis

Cell Type	PC	Protocol	Effect	Ref.
MSC	HP	1% O_2 for 24 h	CXCR4 expression, SDF-1-dependent migration	(Hung et al., 2007)
MSC	HP	3% O_2 for 24 h	CXCR4 and CXCR7 expression, SDF-1-dependent migration, adhesion and survival	(Liu et al., 2010)
PBMNC	HP	2% O_2 for 24 h	CXCR4 expression, adhesion retention	(Kubo et al., 2009)
CLK	HP	0.1% O_2 for 24 h	CXCR4 expression	(Tang et al., 2009)
BM-ckit+ cells	AP	pH 7.0 for 24 h	CXCR4 expression SDF-1-dependent migration	(Cencioni et al., 2013)

MSC: Mesenchymal stem cell PBMNC: Peripheral blood mononuclear cell CLK: cardiosphere-derived, Lin(-)c-kit(+) progenitor, HP: Hypoxic preconditioning AP: Acidosis preconditioning.

Preconditioning of cells via exposure to hypoxia, anoxia, acidosis, low level lasers or other treatments prior to cell injection into the damaged

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tissue, render stem cells more resistant to ischemia (Herrmann et al., 2010).

PC is a promising strategy as it results in the increase of transplanted stem cells recruitment, retention, survival and the induction of a more supportive environment, within the damaged tissue, via secretion of angiogenic factors (Wei et al., 2012). The clinical based outcomes using preconditioning are urgently required. The preconditioning methodologies should be tested carefully in clinical studies, so we could define a better future for stem cell therapy. As PC is a promising approach in the treatment of ischemic heart diseases, the problem which is a major cause of death in our country, optimizing the conditions for PC could greatly reduce the huge life loss.

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