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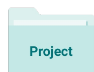

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Effects of Lentiviral Vectors on DNA Damage of Human Dermal Fibroblasts (HDFs)

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In present study we evaluated the DNA damages and cytogenetic stability of transducted and non-transducted human dermal fibroblasts (HDFs) by enhanced green fluorescent protein (eGFP) lentiviral vector using karyotyping, comet assay, and molecular techniques. HDFs were isolated from human foreskin samples and eGFP-expressing lentiviral vector were transfected into HEK-293T cells to produce *lentiviruses*. Then, HDFs at passage 2 were transducted with concentrated eGFP *lentivirus* and transducted HDFs were detected by fluorescent microscope. The expression levels of cell cycle genes include two subunits of anaphase promoting complex (APC) in transducted and non-transducted HDFs were measured by quantitative real-time PCR and finally, karyotype test and comet assay was performed to evaluate the DNA damages and cytogenetic stability in both groups. The results of karyotype analysis were not showed any abnormalities in karyotype of transducted HDFs by eGFP in compared to normal cells. The mean values of alkaline comet assay parameters on non-transducted (normal cells), eGFP-transducted group and positive control (H_2O_2 treatment) were calculated by CaspLab software. The comparison of mean difference of comet assay parameters include tail length, comet length, tail moment, and Olive tail moment by T test between eGFP-transducted HDFs and other groups (positive control and non-transducted HDFs) were statistically significant ($p \leq 0.05$). The alkaline comet assay on HDFs in eGFP-transducted group was showed small tail and indicated slight genetic damage compared with non-transducted group. Furthermore, the analysis of real-time PCR on expression of *APC2* and *APC7* genes in non-transducted HDFs compared with eGFP-transducted HDFs were not significant ($p \leq 0.05$). These findings indicated that integration of lentiviral vectors in first passage of transducted HDFs could not disturb the DNA structure and create chromosome instability. So in genetic engineering and gene transformation these vectors in first passages are useful.

Key words: HDF, *Lentiviruses*, Karyotype, Alkaline comet assay, Molecular technique.

Different types of gene delivery system may be applied in genetic manipulation and gene therapy. In recent decades non-viral and viral gene delivery systems have been developed[1]. Non-viral methods such as physical (microinjection, protoplast fusion, and biolistics transformation), chemical (DEAE-Dextran, polyethene glycol, and calcium phosphate), and electrical methods (electroporation and electrofusion) or any combination of these techniques are used for the

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gene transfer[2]. The disadvantage of non-viral methods are being time-consuming, using a large number of constructs per experiment, low frequency of transformation, and equipments dependent. In viral methods vectors such as *retrovirus*, *adenovirus* (types 2 and 5), adeno-associated virus (AAV), *herpes virus*, *pox virus*, *human foamy virus* (HFV), and more recently *lentivirus* are used[3]. Viral systems produce high gene transfer efficiency with long expression time compare to non-viral systems[4].

Among viral vectors, *lentivirus*es have been proposed as suitable transformation system candidates for gene delivery. *Lentiviruses* have the capacity to infect both proliferating and non-proliferating cells. They can integrate into the host cells genome without incurring cellular toxicity in the absence of an inflammatory response and also they can maintain transgene expression during longer host cell proliferation and differentiation[5]. Despite all remarkable advances, the process of *in vitro* transduction potentially involves complex effects on the cells[5]. Various problems may occur during random integration process of virus into the genome such as impact the epigenetic landscape of the cultured cell population, genotoxicity, and insertional mutagenesis, which may have deleterious effects on the host cell. Significant efforts were made in vector design could improve the biosafety and efficiency of gene transfer. But there are some concerns due to the random insertion of lentiviral vector's DNA into the host genome[6].

As we know, in *lentivirus* transduction, completion of reverse transcription, nuclear import, and genome integration of the transgene occur. Two high risk stages are reverse transcription and genome integration. *Lentivirus* depends on reverse transcriptase to generate a transcription-competent double-stranded DNA template. Reverse transcriptase is error-prone enzyme that may cause mutations in the *lentivirus* genome, including the transgene[5,6].

Engineering of self-inactivating (SIN) lentiviral vectors which lacking the enhancer/promoter unit in the U3 region of the 3'LTR[7,8] could minimize the risk of replication competent of *lentiviruses* and also prevents direct activation of downstream alleles and decreased promoter interference[9].

Insertions at oncogenes and cell-cycle genes are the other concern of lentiviral vectors use for gene transformation. Damage of cell cycle genes and expression of oncogenes may cause chromosome abnormality into the transducted cells. So, cytogenetic evaluations of transducted cells are more important. In recent years, many techniques have been reported for investigation of cytogenetic stability of cells. Comet assay is one of these methods to accuracy assessment of global genomic instability, conducted under alkaline conditions. In comet assay degraded DNA generating a smear that mimics the tail of a comet and show the severity of the DNA damage[10]. Karyotype analysis is another technique which can detect large chromosomal abnormalities such as loss or gain of an entire chromosome or portions of a chromosome and translocations[11]. Furthermore, except cytogenetic techniques cell cycle markers such as p53, p21, and anaphase promoting complex (APC) could use to evaluate genotoxicity of cells which show any problems during mitosis. APC is an E3 ubiquitin ligase and have important role to ubiquitination and subsequent proteasome degradation of multiple cell cycle proteins. Without APC activity, cells cannot separate sister chromatids during anaphase. Therefore, measuring of transcript expression levels of these genes in transducted and non-transducted cells are important to evaluate genetic stability[12,13].

Now a day, lentiviral vector used for gene transformation in most cell types such as neurons, astrocytes, adult neuronal stem cells, induced pluripotent stem cells (iPSc), oligodendrocytes, and glial cells as well as hepatocytes, and antigen-presenting cells (APCs) and mentioned that this vector is a good candidate for gene therapy applications[14,15]. So, in present study we evaluated the cytogenetic stability and DNA damages of human dermal fibroblasts (HDFs) transducted by eGFP-SIN lentiviral vector compared to normal HDFs using karyotyping, comet assay and molecular technique.

MATERIALS AND METHODS

HDFs isolation and cell culture

The foreskin specimens of healthy male newborns were obtained from Kashani Hospital

(Shahrekord City, Iran) and transferred to Cellular and Molecular Research Center. The foreskin tissue was dissected and washed with phosphate-buffered saline (PBS) and centrifuged in 1200 rpm for 5 min. The supernatant was discarded and enzymatic digestions was performed using 0.25 % Trypsin–EDTA solution, 100 U/mL collagenase type IV, and 100 µg/mL of DNase for 20 min. The enzyme activity was neutralized with fetal bovine serum (FBS) and cell suspension was passed through a Mesh filter (BD Falcon, 9340329) and centrifuged at 1200 rpm for 5 min. Then, single cells were cultured at 37°C in a 5% CO_2 atmosphere in Dulbecco's modified Eagle's medium (DMEM) containing 10% FBS, and 1% penicillin/streptomycin antibiotics (all Gibco, Grand Island, NY, USA).

Lentivirus production and concentration

Lentiviruses were produced by co-transfecting HEK-293T cells with an eGFP-expressing *leniviral* vector (Tronolab), gag-pol expression plasmid (psPAX, Tronolab), and VSV-G envelop plasmid (pMDG2, Tronolab) based on the calcium phosphate precipitation principle [10,11]. Briefly, HEK-293T cells were grown on 10 cm plates (Techno Plastic Products (TPP), 93100) to reach 70–80% confluence and then co-transfected with 22.5 µg of eGFP plasmid, 14.6 µg of psPAX and 7.9 µg of pMDG2 in 50 µL of $CaCl_2$ 2.5 mM and 450 µL of 2X HBS (140mM NaCl, 1.5 mM Na_2HPO_4, 50 mM HEPES, pH=7.05) was added and mixed gently and then incubated at room temperature (RT) for 20 min. The mixture was added drop-wise onto the HEK-293T cells. The *lentiviruses* contained supernatant was harvested 48-72 hours post-transfection and centrifuged to remove cell debris, and then were filtered passed through 0.45 µm cellulose acetate filters. Viral supernatants were concentrated by ultracentrifugation at 50,000×g for 2 h to make virus stocks.

Transduction of HDFs with eGFP *lentiviruses*

HDFs at passages 2 were seed in 6 wells plate and were transducted three times with concentrated eGFP *lentiviruses*. The culture media was changed after 12 h by fresh medium. Forty-eight hours after transduction, eGFP positive fluorescent cells were detected by fluorescent microscope (Nikon eclipse E600, Japan).

Karyotype test

Giemsa-banding (G-banding) was performed in triplicate on each eGFP-transducted HDFs and non-transducted HDFs for karyotyping. After cells reach to 80-90% confluence in each well, the culture medium was replaced with media containing 0.1 µg/mL Karyomax Colcemid solution (Cat. no. 15212-012. Invitrogen, Carlsbad, CA, USA) and culture was returned to the CO_2 incubator. After 20 min the cells were trypsinized (0.25 % Trypsin–EDTA solution) and after collection were suspended in 5 mL of 0.075 M KCl solution and incubated in 37°C for 20 min. One mL of cold Carnoy's fixative (methanol/acetic acid, 3:1) was added and mixed with cells and centrifuged at 900 rpm for 10 min at RT. The fixation was performed two times (by added 5 mL fixative and centrifuge at 900 rpm for 10 min). Finally the pellet was resuspended in 200 µL of cold fixative and cells from each suspension were dispensed onto glass slides and baked at 75°C for 3 h. Routine analysis of chromosome G-banding was applied and twenty karyotypes were examined per slide.

Comet assay

In present study alkaline comet assay was down according to standard protocol described by McKelvey-Martin with a few modifications[16]. We used hydrogen peroxide (H_2O_2) treatment as a positive control of comet assay. First, 150×10^3 cells/mL were seeded in 3 cm plate contain DMEM 10% FBS, 2 mM glutamine, and 1% (100 µg/mL) penicillin/streptomycin antibiotics and incubate at 37°C in a CO_2 incubator. Then, the media was replaced by DMEM containing 50 µM H_2O_2 for 60 min to produce massive single-stranded DNA damages and used as an alkaline comet assay positive control. All slides were washed with methanol and heated to remove the proteins. Dakin microscope slides were covered with 250-300 µL of 1% normal melting point agarose (NMA) (Carlsbad, Ca, USA) prepared in PBS at 50°C and were allowed to be fully frosted. To solidify agarose, coverslips was placed on top and the slides were kept on ice. The cells was resuspended in 80 µL of 0.7% low melting point agarose (LMA) (generally at 37°C) and placed directly on a slide. Then, the coverslips were gently removed and cell suspension was pipetted rapidly onto the first agarose layer and the coverslips were replaced on top and the slides left on ice to solidify the agarose. The coverslips gently removed again and the third layer of 250-300 µL of 1% NMA was placed on

slides. As soon as agarose was solidified, the slides were immersed in freshly prepared cold lysis solution (2.5 M NaCl, 100 mM Na_2EDTA, and 10 mM Tris, pH=10, with 1% Triton X-100 and DMSO 10% were added just before use) for 12 h at 4°C. Then, the slides were removed from the lysing solution and after draining placed in a horizontal gel electrophoresis tank side by side. The electrophoresis tank was filled with fresh and ice-cold electrophoresis buffer (300 mM NaOH, 1 mM Na_2EDTA, pH>13) and slides were left in the alkaline buffer for 30 min to allow DNA unwinding occur. Electrophoresis was performed at 25 V (0.66 V/cm) or 300 mA for 30 min at RT. Then, the slides were drained and placed on a tray and flooded slowly with three changes of neutralization buffer (0.4 M Tris, pH=7.5) for 5 min to remove alkali and detergents. All slides just once washed with ethanol 95% for 5 min and were drained. The slides were stained with 2 µg/mL of ethidium bromide and placed into humidified chamber at 4°C. All slides were visualized under UV light and duplicate slides for each treatment were prepared. The parameters of alkaline comet assay include head area, tail area, head DNA, tail DNA, tail length, comet length, tail moment, and Olive tail moment of positive control, non-transducted and eGFP-transducted HDFs were evaluated by CaspLab (Comet Assay Software Project) software version 1.0.0.

Reverse transcriptase PCR

Total RNA of HDFs in two groups was isolated using BIOZOL Kit (BSC51M1) according to the manufacturer's instructions. The total extracted RNA was measured by NanoDrop ND-1000 (PeqLab) according to the method described by Sambrook and Russell[17]. The cDNA was synthesized from 1 µg of total RNA using the PrimeScript™ RT Reagent Kit (Takara Bio Inc, RR037A) at 37°C for 15 min to the reverse transcription, and at 85°C for 5 s to inactivate the reverse transcriptase. The specific primers used in this study for gene amplification were designed using PERL primer software and the sequences are shown in Table 1. For gene amplification PCR reaction were performed in a final volume of 25 µL in 0.5 mL tubes containing 1 µg of template cDNA, 0.25 µM of each primer, 2 mM $MgCl_2$, 200 µM dNTP mix, 2.5 µL of 10X PCR buffer (20 mM Tris–HCl pH=8.4 and 50 mM KCl), and 1 unit of *Taq* DNA polymerase (Roche Applied Science, Germany). The samples were amplified in a Gradient Palm Cycler (Corbett Research, Australia). PCR temperature programs involved an initial denaturation at 94°C for 5 min; followed by 35 cycles at 94°C for 30 s, annealing at 60°C for 30 s, extension at 72°C for 30 s, and a final extension at 72°C for 5 min was done at the end of the amplification.

The amplified cDNA products were detected in 2% agarose gel electrophoresis. The electrode buffer was TBE (10.8 g of Tris-base 89 mM, 5.5 g of Boric acid 2 mM, 4 mL of 0.5 M EDTA (pH=8.0), combine all components in sufficient H_2O and stir to dissolve). Aliquots 10 µL of PCR products were applied to the gel. Constant voltage of 80 V for 30 min was used for products separation. The 50 bp DNA ladder (Fermentas, Germany) was used as a molecular weight marker to determine the length of the amplified fragments. After electrophoresis, the gel was stained with ethidium bromide and examined under UV light and photographed were obtained in UVIdoc gel documentation systems (Uvitec, UK).

Real-time PCR assay

Relative transcript expression levels of eGFP-transducted and non-transducted HDFs were measured by quantitative real-time PCR using a SYBR Green master mix (Roche Applied Science, Indianapolis, IN, USA) based method. The real-time PCR performed in 10 µL reaction contained: 5 µL of SYBR Green master mix, 2.5 nM concentration of each forward and reverse primer, and 60 ng/µL of cDNA sample. Averages of fold changes were calculated by differences in threshold cycles (Ct) between samples and GAPDH gene (internal control). Then, micro-tubes were placed into Rotor-Gene 3000 (Corbett, Australia) for gene amplification. The reaction program was as follows: an initial denaturation step at 95°C for 10 min, 45 cycles includes denaturation at 95°C for 10 s, annealing at 60°C for 20 s, and extension at 72°C for 20 s. The experiments were carried out in duplicate for each data point. The relative quantification in gene expression was determined using the $2^{-\Delta\Delta Ct}$ method. GAPDH was used as internal control to normalize and a melting curve acquired by heating the products to 95°C, cooling to 55°C and maintaining at 70°C.

Ethical approval

In this study the protocols and informed consent forms were approved by the Regional

Research Ethical Committee of Shahrekord University of Medical Sciences (Grant No. 91-12-14). Foreskin specimens of healthy male newborns were obtained and consent forms were filled by parents of each infant. Male newborns with genetic disorders such as Down's syndrome were excluded from the study.

Statistical analysis

The alkaline comet assay parameters were evaluated by CaspLab software version 1.0.0 and all data were collected in Statistics programs for the Social Sciences software (SPSS, Inc., Chicago, IL, USA) version 17. The mean difference between groups was calculated by T test. In this study *p*-value of d"0.05 was considered statistically significant.

RESULTS

HDF cells preparation

HDFs isolated from human foreskin samples after 5 days were ready to first passage and in second passage the cells were used for *lentivirus* transduction. To evaluate the cytogenetic stability and DNA damages of transducted and non-transducted HDFs karyotype test and comet assay were used. Figure 1 shows the HDFs isolated from human foreskin sample.

HEK-293T cell transfection and virus production

We transfected HEK-293T cells with a lentiviral plasmid expressing eGFP by *$CaPO_4$ precipitation* method and used produced *lentiviruses* at a multiplicity of infection (MOI) of

Table 1. The sequence of primers used for gene amplification

Primersname	Sequence	Product length (bp)	GenBank accession number
APC2-F	5' -ATGCAGAGTAAGAGGAAGGG-3'	200	NM_013366
APC2-R	5' -CTGAGCCTGTGTAAGACCTG-3'		
APC7-F	5' -CCATTCTAGGCTTGTTGTCCC-3'	200	NM_016238
APC7-R	5' -CCACGTTATCTCGCAATAAGG-3'		
GAPDH-F	5' -CTCATTTCCTGGTATGACAACGA-3'	121	NM_001256799
GAPDH-R	5' -TTCCTCTTGTGCTCTTGCTG-3'		

Table 2. The details of alkaline comet assay on non-transducted (normal cells), eGFP-transducted HDFs, and positive control (cells exposed to H_2O_2)

Parameters	eGFP-transducted HDFs No. of cells (100) Mean ± SD	Normal cellsNo. of cells (100) No. of cells (100) Mean ± SD	H_2O_2 treatment Mean ± SD
Head Area	780.78 ± 187.277	321.86 ± 54.21	433.04 ± 186.844
Tail Area	342.58 ± 72.511	38.58 ± 15.94	1809.58 ± 426.41
Head DNA	47.31 ± 8.111	32.73 ± 4.733	24.12 ± 13.28
Tail DNA	7.17 ± 1.618	0.96 ± 0.44	51.98 ± 23.54
Head DNA %	86.93 ± 1.41	97.14 ± 1.3	30.62 ± 3.783
Tail DNA %	13.07 ± 1.41	2.86 ± 1.3	69.38 ± 3.783
Tail Length	11.66 ± 3.33	4.20 ± 1.97	44.10 ± 5.97
Comet Length	43.72 ± 4.68	24.96 ± 3.01	67.78 ± 7.755
Tail Moment	1.56 ± 0.6	0.13 ± 0.123	30.51 ± 3.995
Olive Tail Moment	2.28 ± 0.39	0.27 ± 0.139	18.67 ± 2.265

Head Area: Area of the comet head in pixels, Tail Area: Area of the comet tail in pixels, Head DNA: Amount of DNA in the comet head, Tail DNA: Amount of DNA in the comet tail, Tail Length: Length of the comet tail measured from right border of head area to end of tail (in pixels), Comet Length: Length of the entire comet from left border of head area to end of tail (in pixels), Tail Moment: Tail DNA% x Tail Length ([percent of DNA in the tail] x [tail length]), Olive Tail Moment: Tail DNA% x (Tail Mean X - Head Mean X) ([percent of DNA in the tail] x [distance between the center of gravity of DNA in the tail and the of center of gravity of DNA in the head in x-direction]), SD: Standard deviation

Table 3. The comparison of alkaline comet assay parameters on HDFs transducted by eGFP, non-transducted (normal cells), and positive control (H_2O_2 treatment) by T test

Parameters	eGFP-transducted HDFs compare with positive control (H_2O_2)		eGFP-transducted HDFs compare with normal cells	
	Mean difference* ± SE	*p*-value	Mean difference* ± SE	*p*-value
Head Area	347.74 ± 26.454	0.915	-458.920 ± 19.496	0.000
Tail Area	-1467 ± 43.253	0.000	-304 ± 7.424	0.000
Head DNA %	56.302 ± 0.404	0.000	10.214 ± 0.191	0.405
Tail DNA %	-56.302 ± 0.404	0.000	-10.214 ± 0.191	0.405
Tail Length	-32.44 ± 0.683	0.000	-7.46 ± 0.387	0.000
Comet Length	-24.06 ± 0.91	0.000	-18.76 ± 0.557	0.000
Tail Moment	-28.96 ± 0.404	0.000	-1.425 ± 0.06	0.000
Olive Tail Moment	-16.39 ± 0.23	0.000	-2.015 ± 0.041	0.000

*The mean difference is significant at $p < 0.05$.
SE: Standard error

10 (Fig. 2). Then, the human fibroblasts were transducted. After 72 h the results were showed high efficiency of eGFP expressing in transducted HDFs (Fig. 3).

Karyotype test

G-banding was conducted to determine the karyotype of eGFP-transducted and non-transducted HDFs groups. eGFP-transducted HDFs were passaged one time before karyotype evaluation. Twenty karyotypes per each slide were analyzed. We showed that eGFP-transducted HDFs had normal karyotype (20/20, 46 chromosomes, XY) same to normal group (Fig. 4 and 5). The karyotype analysis was not showed any abnormalities including deletions, insertions, and duplications in karyotypes of transducted HDFs by eGFP compared to normal cells.

Comet assay

Alkaline comet assay was performed on 100,000 HDFs in each three groups including non-transducted (healthy cells), eGFP-transducted, and HDFs exposed to H_2O_2. The H_2O_2 treatment was used for DNA damage of HDFs as positive control in comet assay (Fig. 6A). Alkaline comet assay of eGFP-transducted HDFs group were showed small tail, and little migration of DNA fragments (Fig. 6B and 6C). These results were indicated slight genetic damage compared with non-transducted group (without comet tail and DNA damages and genetic material remain inside the nucleus).

The mean values of alkaline comet assay parameters include head area, tail area, head DNA, tail DNA, tail length, comet length, tail moment, and Olive tail moment in non-transducted HDFs (normal cells), eGFP-transducted group (passage 2) and positive control (H_2O_2 treatment) were calculated by CaspLab software version 1.0.0 (Table 2). The amount of DNA damages of cell was estimated from tail length as the extent of genetic material migration in the direction of anode electrode. Tail moment was measured by the percent DNA in the tail multiplied by the distance between the means of the head and tail distribution. So, Olive tail moment was best comet descriptor to evaluate low DNA damage levels. All date were collected in SPSS software and relationship and analysis of mean difference between groups were evaluated by T test (Table 3).

The comparison of mean difference of comet assay parameters include tail length, comet length, tail moment, and Olive tail moment by T

Fig. 1. HDFs isolated from human foreskin samples in passage 2

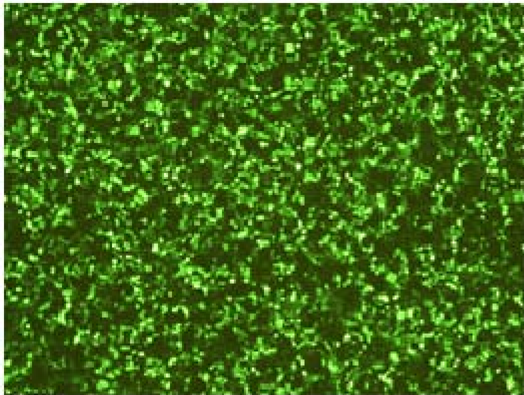


Fig. 2. HEK-293T cells transfected with lentiviral plasmid expressing eGFP after 48 h

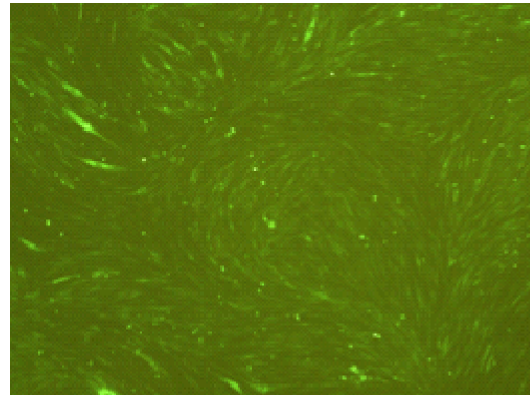
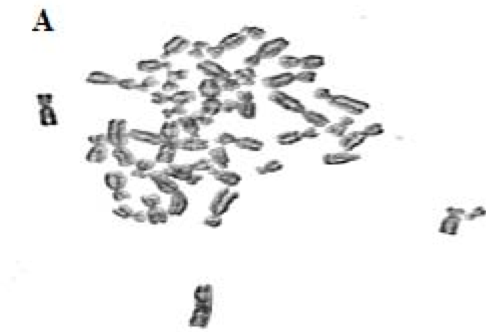


Fig. 3. HDFs transducted by eGFP *lentiviruses* after 72 h



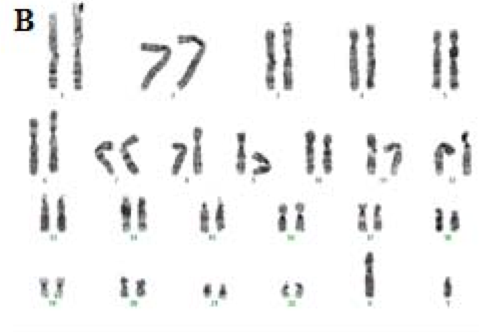
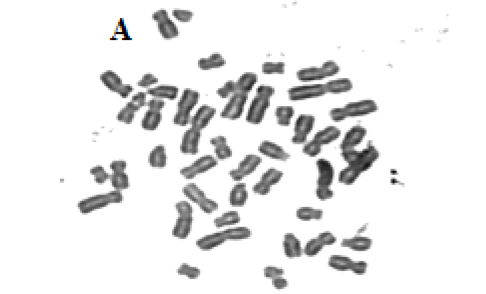


Fig. 4. A) The metaphase and B) karyotype of normal HDF



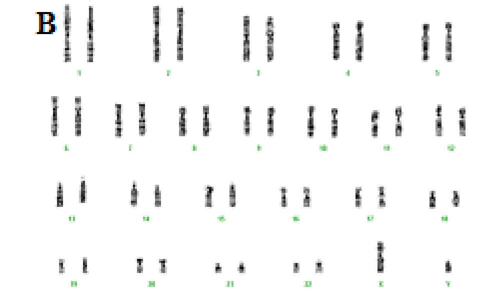


Fig. 5. A) The metaphase and B) karyotype of eGFP-transducted HDF after first passage

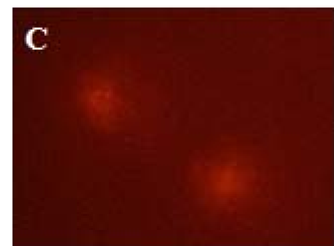


Fig. 6. A) The alkaline comet assay of HDFs treated by H_2O_2 (positive control) to create DNA damage. B) Alkaline comet assay of non-transducted (normal cells) and C) eGFP-transducted HDFs (slight genetic damage)

test between eGFP-transducted and non-transducted HDFs as well as positive control were statistically significant ($p \leq 0.05$).

Conventional reverse transcriptase PCR analysis

Amplification of cDNA samples with specific oligonucleotide primers on 2% agarose gel electrophoresis revealed a 200, 200, and 121 bp fragments for *APC2*, *APC7*, and *GAPDH* (internal control) genes, respectively (Fig. 7).

Real-time PCR analysis

The analysis of Real-time PCR results on expression of *APC2* (0.000077358 vs. 0.000116855, $p<0.517$) and *APC7* (0.015102575 vs. 0.002185813, $p<0.156$) genes in non-transducted HDFs compared with eGFP-transducted HDFs were not statistically significant. All data were normalized by endogenous GAPDH expression and calculated with $2^{-\text{"}CT}$. In current study both genes were not up regulated during virus transduction compared with non-transducted HDFs.

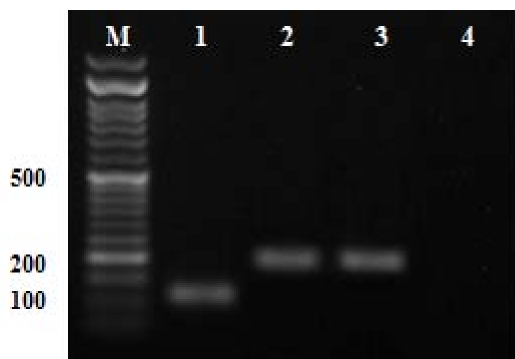


Fig. 7. The agarose gel electrophoresis of reverse transcriptase PCR products amplified by specific oligonucleotide primers (Line M is 50 bp molecular weight marker (Fermentas, Germany), line 1 is *GAPDH* (internal control), lines 2 and 3 are *APC2* and *APC7* fragments, respectively, and line 4 is negative control (no DNA)

DISCUSSION

Lentiviral vectors are a successful candidate for gene delivery that can crossing the nuclear membrane and integrate their genome into host cells. Insertion of lentiviral vectors are randomness and integration site analysis show that these insertion are preferred to gene-rich areas of chromosomes that can disrupt existing normal genes and increase the genotoxic risk such as insertional mutagenesis[18,19]. So, in present study we evaluated cytogenetic stability of HDFs during lentiviral vector integration *in vitro*. For our purpose karyotype analysis and alkaline comet assay performed to determine the HDFs cytogenetic instability in the first passage after transducted with SIN eGFP *lentivirus*. Our findings indicated that HDFs had normal karyotype after eGFP transduction like non-transducted HDFs. The alkaline comet assay of transducted-HDFs showed short tail but not considerable (*p*e"0.05). Although in these groups were shown small tail but in comet assay the chromatin structure could damage after alkali denaturation and renaturation. Also, single-strand breaks in replication forks of S-phase may occur. So, low tail moment in healthy cells is not considerable[20,21,22,23]. In parallel the differences expression of the cell cycle regulatory genes between transducted and non-transducted HDFs are not significant.

Focusing on the architecture lentiviral plasmid to optimize vector design could reduce genotoxic risk of integrating. The important concerns via vector integration are insertional inactivation of cell cycle genes and tumor suppressor genes which cause genome instability and finally apoptosis. Some effort such as self inactivating (SIN), lineage-specific or regulatable promoters and drug-inducible systems have been done to overcome these genotoxic events. For example, in SIN design elimination of enhancer sequences reducing of interaction with neighbor or long distance genes could decrease the insertional transformation in target cells and also reduce the risk of vector mobilization[24]. Moreover, elevation of vector copy numbers can increases the risk of insertional oncogenesis. So, vector designs could reduce copy numbers per cell in experiments to achieve optimum expression level[25]. Furthermore, lentiviral vectors could accommodate large transgenes (up to ~10 kb) and decrease vector titers with large inserts compared to other types of viruses such as *retroviruses*[26]. So in current study SIN lentiviral vector was used and there was no change in the cytogenetic analysis.

Previous study on Cdkn2a–/–mice (which are susceptible to a broad range of cancer-triggering genetic lesions) showed that insertions at oncogenes and cell-cycle genes enriched in early-onset tumors, and this phenomenon more occurred for retroviral vectors compare to lentiviral

vectors[27]. This finding indicate that the SIN lentiviral vector have low oncogenic, genotoxic potential and did not accelerate the rate of tumor formation compare to other viral vectors[27,28]. In the other hand *in vitro* studies by cell immortalization assays on several types of viral vectors showed that integration of *lentiviruses* and derived vectors into transcribed regions of active genes are more frequent, while in promoter-proximal regions are low. So, these findings indicated that incidence and severity of insertional side effects on the neighboring genes are reduced. But the study of Pellegrini and their co-workers indicted that keratinocyte stem cells can be stably transduced with retroviral vectors and these cells are attractive targets for the gene therapy of genodermatoses[29]. Another study on several types of viral vectors showed that integration of *lentiviruses* and derived vectors into transcribed regions of active genes are more frequent, while in promoter-proximal regions are low. So, these findings indicated that incidence and severity of insertional side effects on the neighboring genes are reduced[30,31]. In the other hand, co-insertion in both alleles of a gene would be rare and disruption of clonal homeostasis is very low[32]. In comet assay the chromatin structure could damage after alkali denaturation and renaturation. Also, single-strand breaks in replication forks of S-phase may occur. So, low tail moment in healthy cells is not considerable[33,34]. Therefore, cell cycle checkpoints must be evaluated by expression of cell cycle regulator genes such as anaphase-promoting complex/cyclosome (APC/C), p53 and p21. The DNA lesions can activate these regulator genes and arrest cell cycle. APC is a multi-protein complex which composed of at least 12 subunits with E3 ubiquitin ligase that controls the cell cycle and it is required for cell cycle control and active from early mitosis through late G1[12]. Previous studies indicated that DNA damage activates APC in a p21-dependent manner induced G2 arrest by APC activation. So, in current research we evaluated the expression of two main subunits of APC includes *APC2* and *APC7* genes[23]. Activation of the APC/C during S or G_2 phases may result in the degradation of regulators that promote cell cycle. This would lead to cell cycle arrest and allow time for the cell to repair the damaged DNA and prevent replication or segregation of damaged DNA[35,36,37,38]. So, DNA damage caused by the *lentiviruses* can up-regulate APC expression to arrest G2[23]. The results of present study not showed any over-expression in eGFP-transducted HDFs compare to non-transducted group and indicated no DNA damage and progression of normal cell division.

CONCLUSIONS

The advantage of lentiviral vector technology not only in its efficiency for stable gene delivery but also is remarkable candidate as a research tool in over expression or knock down of desired gene. Due to the great efforts in developing of safer vectors for gene manipulation and despite considerable progress, quantitative assays are requiring to evaluate any vector genomic side effect. Our findings indicated that integration of lentiviral vector after first passage of transduced-HDFs cannot disturb genome and chromosome stability. Therefore, lentiviral vectors could be safe for gene delivery in primary passages and useful for gene transformation and gene manipulation research in cell-culture system.

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