Original Article
Middle East Journal of Cancer; April 2018; 9(2): 113-122

The Effect of Placenta Growth Factor Knockdown on hsa-miR-22-3p, hsa-let-7b-3p, hsa-miR-451b, and hsa-mir-4290 Expressions in MKN-45-derived Gastric Cancer Stem-like Cells

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Abstract

Background: Placental growth factor is involved in human gastric cancer initiation and progression through stimulating the proliferation, angiogenesis, invasion and metastasis of cancerous cells. Previous studies indicate that the expression profiles of hsa-miR-22-3p, hsa-let-7b-3p, hsa-miR-451b, and hsa-mir-4290 change in MKN-45-derived gastric cancer stem-like cells. Therefore, this study aims to investigate the effect of *PlGF* knockdown on hsa-miR-22-3p, hsa-let-7b-3p, hsa-miR-451b, and hsa-mir-4290 expressions in MKN-45-derived gastric cancer stem-like cells.

Methods: We used a non-adhesive culture system to derive the cancer stem-like cells from MKN-45 cells. *PlGF* gene silencing was performed by *PlGF*-specific siRNA. The transcript of *PlGF* and miRNAs were measured by real-time RT-PCR. We conducted bioinformatics analyses with the online software programs TargetScan, miRanda, miRWalk, PicTar, and the Database for Annotation, Visualization, and Integrated Discovery tools to predict miRNAs' targets and their signaling pathways.

Results: hsa-let-7b-3p had a 2.28-fold up-regulation, whereas we observed down-regulation of hsa-mir-451b (25%), hsa-mir-4290 (34%), and hsa-mir-22-3p (9%). Bioinformatics analysis results indicated that the miRNA target genes TGF-β, MAPK, and Wnt, and hedgehog signaling pathways contributed to cancer initiation and progression by influencing different cellular behaviors.

Conclusion: We suggest that *PlGF* signaling may influence miRNA expression profiles in MKN-45-derived cancer stem-like cells, which can influence the expressions of different genes and signaling pathways. However, more empirical studies should determine the exact effect of *PlGF* knockdown on the expression of miRNA targets in cancer stem-like cells to locate their actual gene targets.

Keywords: *PlGF*, Cancer stem cell, miRNA, MKN-45

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Introduction

Placental growth factor (*PlGF*) is a member of the vascular endothelial growth factor (VEGF) family that induces invasion, metastasis, tumor progression and survival of gastric

Received: May 29, 2017; Accepted: October 17, 2017

cancer cells.[1,2] Placental growth factor promotes the growth, survival, angiogenesis, invasion, and migration of cancerous cells.[3] It directly stimulates vessel growth and maturation by affecting stromal cells and recruitment of pro-angiogenic cell types.[4] It has been found that *PlGF* is related to angiogenic and inflammatory signaling in cancer.[3] A distinct population of cells in tumors, cancer stem cells (CSCs), express *PlGF* to increase cancer invasion and progression.[5] Cancer stem cells play a significant role in cancer development through stimulation of malignancy, angiogenesis, and tumor growth.[6,7]

MicroRNAs (miRNAs) are a class of endogenous, small non-coding RNAs that play important roles in post-transcriptional regulation.[8] The miRNA expression profile undergoes changes in many cancers, including gastric cancer. Depending on the target genes, they act as oncogenes or tumor suppressors.[9,10] Previous studies have indicated that miRNAs exhibit a high potential for regulating the biological functions of CSCs such as proliferation, differentiation, and tumor formation by targeting molecules involved in CSC signaling pathways.[11] Many signaling pathways such as Wnt/β-catenin, notch, sonic hedgehog homolog (SHH), mitogen-activated protein kinase (MAPK), mammalian target of rapamycin (mTOR), and Janus kinase/signal transducer and activator of transcription (JAK-STAT) participate in the regulation of CSC growth and function.[12-16] Studies have shown that miRNAs regulate these signaling pathways in cells. Changes in the expression profiles of miRNAs contribute to cancer initiation and progression.[17-18] In this regard, previous studies have shown significant changes in the expression profiles of hsa-miR-22 and hsa-let-7b/g in gastric CSCs. They could act as tumor suppressors in gastric cancer.[19,20] Microarray data also indicated that both hsa-miR-451b and hsa-mir-4290 up-regulated in the cancer stem-like cells (CSLCs) derived from the MKN-45 cell line.[21] Validated pathway-gene-miRNA interactions by miRWalk tools indicated that hsa-mir-22-3p was involved in angiogenesis signaling pathway by targeting Wnt1, HIF1A, and PIK3C2A. hsa-mir-451b plays an important role in angiogenesis pathway by targeting FGF2 and VEGFA mRNAs. In addition, APC2 and CRKL are targeted by hsa-mir-4290 and these target genes play role in the angiogenesis signaling pathway. hsa-let-7b-3p participates in the angiogenesis signaling pathway by targeting FZD5 and GSK3B mRNAs.[22]

We took into consideration the fact that *PlGF* signaling pathway has an important role in cancer initiation and progression, in addition to the roles of hsa-mir-22-3p, hsa-let-7b-3p, hsa-mir-451b, and hsa-mir-4290 in these processes. Hence, in this study, we aimed to evaluate the effect of *PlGF* knockdown on the expressions of these miRNAs at the transcript level in MKN-45-derived gastric CSLCs. In addition, we used bioinformatics analysis to predict competent targets of miRNAs.

Materials and Methods

Cell culture

We obtained the human gastric cancer cell line, MKN-45, from the National Cell Bank of Iran (NCBI), Pasteur Institute, Tehran, Iran. MKN-45 cells were cultured in DMEM/F12 medium supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin G, and 100 µg/mL streptomycin at 37ºC in the presence of a humidified atmosphere with 5% CO_2. The culture media were changed twice a week.

Derivation of cancer stem-like cells (CSLCs) from MKN-45 cells

MKN-45 cells (5×10^4 cells) were dissociated into single cells and seeded in DMEM/F12 medium supplemented with 10% FBS on the non-adhesive surfaces of 10 cm^2 culture plastic dishes coated with 1.5% agarose gel. The plates were incubated at 37ºC in a humidified incubator that contained 5% CO_2 for two weeks. The culture medium was changed every three days until spheroid body cell formation.

Table 1. MicroRNA (miRNA) sequences.

miRNA length	miRNA sequence	miRNA
22 nt	CUAUACAACCUACUGCCUUCCC	Hsa-let-7b-3p
22 nt	AAGCUGCCAGUUGAAGAACUGU	Hsa-mir-22-3p
22 nt	UAGCAAGAGAACCAUUACCAUU	Hsa-mir-451b
19 nt	UGCCCUCCUUUCUUCCCUC	Hsa-mir-4290

siRNA knockdown of placental growth factor (PlGF)

The single-cell suspensions obtained by dissociation of CSLCs were plated at a 1×10^6 seeding density in DMEM/F12 that contained 5% FBS on 10 cm^2 culture plastic dishes with non-adhesive surfaces. The cells were incubated at 37°C, 95% humidity, and 5% CO_2. The spheroid bodies at 50%-60% confluency were transfected with 40 pmols of *PlGF*-specific siRNA and 40 pmols of scrambled siRNA as the control (Santa Cruz Biotechnology, Inc.).

Primer designs for microRNAs (miRNAs)

We obtained the miRNA sequences from http://www.mirbase.org (Table 1). In this study, we used the stem-loop method to design specific primers for the miRNAs. Specific stem-loop RT primers comprised 44 nt of the stem-loop sequence according to Chen et al., with the six 3′nt of the desired mature miRNAs.[23] Forward primers included the first 12 nt from the 5′end of the mature miRNA sequences and a 6 additional nt at 5′end of the primers to obtain the Tm to 60±1°C. A universal primer with 16 nt of the stem-loop sequence in RT primers was used as a reverse primer (Table 2).

RNA extraction and reverse transcriptase reactions

Total RNA was extracted from the CSLCs treated with *PlGF*-specific siRNA and scrambled siRNA using the Qiagen RNeasy Mini kit (Qiagen) according to the manufacturer's instructions. The purification and integrity of the RNA samples were monitored by agarose gel electrophoresis. The RNA reverse transcription reaction was carried out with the QuantiTect® Reverse Transcription kit (Qiagen) according to the manufacturer's protocol.

Quantitative real-time PCR

Quantitative gene expression was accomplished with the Quanti Tect™ SYBR® Green PCR kit (Qiagen, USA) and a Rotor-Gene 3000 system (Corbett Research, Australia). The amplification efficiency of the reactions was determined with a two-fold serial dilution series and construction of a standard curve. Real-time PCR data analysis was performed according to the $2^{-\Delta\Delta Ct}$ method and target genes were normalized based on the glyceraldehyde-3' phosphate dehydrogenase (GAPDH) as an endogenous control gene. Each experiment was repeated at least twice and in triplicate.

Target prediction and bioinformatics analysis

Four online software programs, TargetScan (http://www.targetscan.org), miRanda algorithm (http://www.microrna.org/), miRWalk (http://zmf.umm.uni-heidelberg.de/apps/zmf/mirwalk2/), and PicTar (http://pictar.mdc-berlin.de/cgi-bin/PicTar_vertebrate.cgi) were used to predict hsa-miR-22-3p, hsa-mir-451b, hsa-mir-4290, and hsa-let-7b-3p targets. We used the Database for Annotation, Visualization, and Integrated Discovery (DAVID) tools (http://www.david.abcc.ncifcrf.gov/), KEGG, BIOCARTA, Reactom, and Panther pathways to analyze the signaling pathways of the target miRNA genes.

Statistical analysis

All experiments were performed in two or three independent triplicate tests. Data analyses were performed using SPSS version 16.0 for Windows. The differential expression of each assay was evaluated by the Student's t-test and presented as the mean ± SEM. *P*-values less than 0.05 were considered statistically significant.

Table 2. The sequences of stem-loop RT, forward and reverse primers and PCR product lengths.

Gene ID	Primer/ product length (bp)	Sequence (5ˈ to 3ˈ)
Hsa-let-7b-3p	RT primer	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACGGGAAG
	Forward primer	CGCGCTATACAACCTACTGCCT
	Reverse primer	GTGCAGGGTCCGAGGT
	Product Length	70
Hsa-mir-22-3p	RT primer	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACACAGTT
	Forward primer	ACGCAAGCTGCCAGTTGAAG
	Reverse primer	GTGCAGGGTCCGAGGT
	Product Length	70
Hsa-mir-451b	RT primer	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACAATGGT
	Forward primer	CGCGCTAGCAAGAGAACCATTA
	Reverse primer	GTGCAGGGTCCGAGGT
	Product Length	71
Hsa-mir-4290	RT primer	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACGAGGGA
	Forward primer	CGCTGTATCAGGGAGTCAGG
	Reverse primer	GTGCAGGGTCCGAGGT
	Product Length	69
GAPDH	RT primer	Random hexamer
	Forward primer	ACTCTGGTAAAGTGGATATTGTTGC
	Reverse primer	GGAAGATGGTGATGGGATTTC
	Product Length	162
PlGF	RT primer	Random hexamer
	Forward primer	CGGCTCGTCAGAGGTGGAAG
	Reverse primer	GCAGGGAGACACAGGATGG
	Product Length	143---

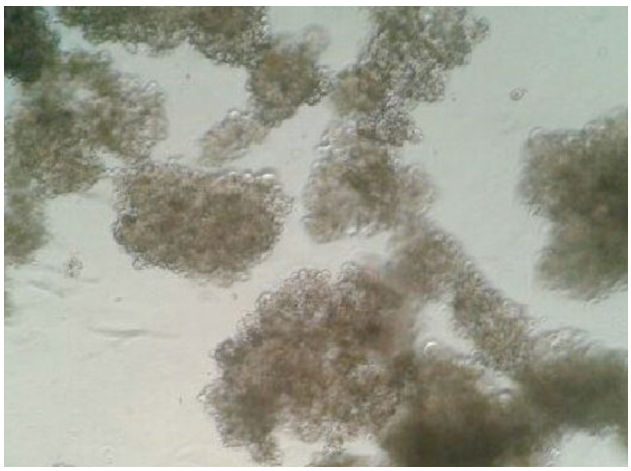

Figure 1. Derivation of cancer stem-like cells (CSLCs) from MKN-45 cells. Spheres were derived from MKN-45 cells by seeding 5×10^4 cells in DMEM/F12 medium supplemented with 10% fetal bovine serum (FBS) on the non-adhesive surfaces of 10 cm^2 culture plastic dishes coated with 1.5% agarose gel.

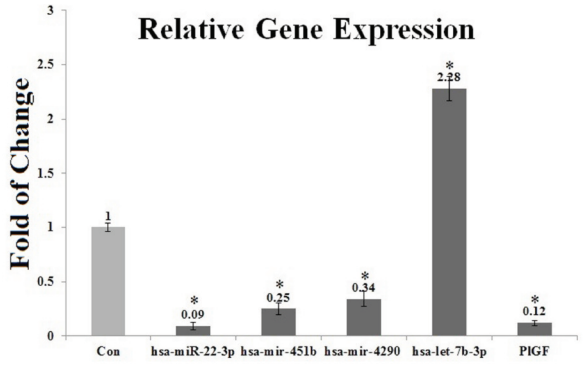

Figure 2. The effects of placental growth factor (*PlGF*)-specific siRNA on the expressions of *PlGF*, hsa-mir-22-3p, hsa-mir-451b, hsa-mir-4290, and hsa-let-7b-3p. Down-regulation of *PlGF* in cancer stem-like cells (CSLCs) derived from MKN-45 compared to untreated CSLCs derived from MKN-45 cells as the control. Cancer stem-like cells treated with 40 pmol of *PlGF*-specific siRNA decreased the transcript levels of *PlGF* (12%), hsa-miR-22-3p (9%), hsa-mir-451b (25%), and hsa-mir-4290 (34%). However, the transcript level of hsa-let-7b-3p increased by 2.28-fold. Results were shown as mean± S.E.M. of three independent experiments in triplicate.

Results

Cancer stem-like cells (CSLCs) derived from MKN-45 cells

MKN-45 cells were dissociated into single cells and seeded into culture plastic dishes that had non-adhesive surfaces. At 2-3 days after cell seeding, a number of the cultured cells underwent apoptosis in non-adhesive surface. Cancer stem-like cells formed colonies after 15 days (Figure 1).

Placental growth factor (PlGF) knockdown by PlGF-specific siRNA

The spheroid body-forming cells were transfected with 40 pmols of *PlGF*-specific siRNA. Quantitative real-time RT-PCR results of *PlGF* knockdown revealed that *PlGF*-specific siRNA decreased the level of the *PlGF* gene to 12% compared to scrambled siRNA (Figure 2).

Differential expression of hsa-mir-22-3p, hsa-mir-451b, hsa-mir-4290 and hsa-let-7b-3p in cancer stem-like cells (CSLCs) treated with placental growth factor (PlGF)-specific siRNA

We used quantitative real-time RT-PCR to examine the effects of *PlGF*-specific siRNA on expressions of hsa-mir-22-3p, hsa-mir-451b, hsa-mir-4290, and hsa-let-7b-3p in MKN-45-derived CSLCs compared to the MKN-45 cells. Down-regulation of *PlGF* decreased the expression levels of hsa-miR-22-3p (9%), hsa-mir-451b (25%), and hsa-mir-4290 (34%; $P<0.05$) in siRNA-treated CSLCs compared to the control. However, the hsa-let-7b-3p transcript up-regulated by 2.28-fold (Figure 2).

Target prediction and signaling pathway analysis

We identified target genes of the miRNAs by four online software programs - Targetscan, miRanda, PicTar, and miRWalk. Our results indicated that some miRNAs have dual roles in cancer progression as oncogenes and tumor suppressor genes. For example, one of the hsa-mir-22-3p target genes, Fas (TNF receptor superfamily, member 6), regulated the p53 signaling, apoptosis signaling, and MAPK signaling pathways. UBE2E1, as a target of hsa-mir-451b, regulated PDGF, Ras, Wnt, TGF-β, and Notch signaling pathways, and influenced cellular behaviors such as the cell cycle and angiogenesis. In addition, the hsa-mir-4290 target genes participated in regulation of TGF-β, Wnt, and hedgehog signaling pathways, melanogenesis, and formation of basal cell carcinomas. Bioinformatics results also indicated that hsa-miR-22-3p, hsa-let-7b-3p, hsa-miR-451b, and hsa-mir-4290 participated in cell signaling pathways such as CSCs-related signaling pathways. Table 3 and figure 3 show the target genes and signaling pathway for hsa-mir-4290.

Discussion

Understanding the signaling pathways and molecules involved in the pathogenesis of cancer cells can improve the efficacy of anticancer therapeutic approaches. MicroRNAs have a high regulatory potential in the biological function of CSCs by targeting molecules involved in CSC signaling pathways.[11] Liu et al. have analyzed the miRNA expression profile of gastric MKN-45-derived CSCs. They showed that 9 miRNAs over-expressed in the CSCs, while 173 miRNAs under-expressed.[21] In a previous study, we observed that *PlGF* silencing induced apoptosis and decreased cell proliferation, migration ability,

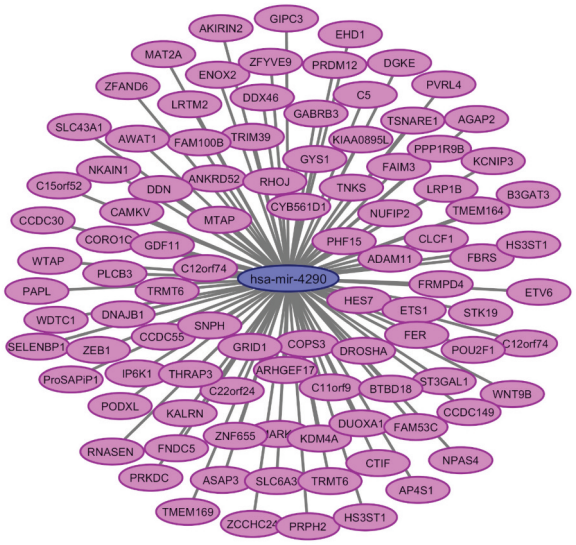

Figure 3. The predicted signaling pathways for the microRNAs (miRNAs). hsa-mir-4290 participated in cell signaling pathways such as cancer stem cell (CSCs)-related signaling pathways.

Table 3. Target genes and signaling pathways for hsa-mir-4290.

KEGG PATHWAY	Endocytosis
814582	COP9 constitutive photomorphogenic homolog subunit 3 (Arabidopsis)
PANTHER PATHWAY	P00013: Cell cycle, P00060: Ubiquitin proteasome pathway
797388	DnaJ (Hsp40) homolog, subfamily B, member 1
BIOCARTA	Hypoxia and p53 in the cardiovascular system, prion pathway
782172	EH-domain containing 1
KEGG PATHWAY	Endocytosis
816442	Kv channel interacting protein 3, calsenilin
BIOCARTA PATHWAY	Repression of pain sensation by the transcriptional regulator DREAM
799640	POU class 2 homeobox 1
REACTOME PATHWAY	REACT_1788: Transcription
802230	Rho guanine nucleotide exchange factor (GEF) 17
REACTOME PATHWAY	REACT_11044: Signaling by Rho GTPases
807496	ST3 beta-galactoside alpha-2,3-sialyltransferase 1
KEGG PATHWAY	O-Glycan biosynthesis, keratin sulfate biosynthesis, glycosphingolipid biosynthesis, glycosphingolipid biosynthesis
806007	Beta-1,3-glucuronyltransferase 3 (glucuronosyltransferase I)
KEGG PATHWAY	Chondroitin sulfate biosynthesis, heparan sulfate biosynthesis
779476	Cardiotrophin-like cytokine factor 1
KEGG PATHWAY	Cytokine-cytokine receptor interaction, Jak-STAT signaling pathway
794042	Diacylglycerol kinase, epsilon 64kDa
KEGG PATHWAY	Glycerolipid metabolism, glycerophospholipid metabolism, phosphatidylinositol signaling system
775529	Ets variant 6
KEGG PATHWAY	Dorso-ventral axis formation
783149	Gamma-aminobutyric acid (GABA) A receptor, beta 3
KEGG PATHWAY	Neuroactive ligand-receptor interaction
774841	Glutamate receptor, ionotropic, delta 1
KEGG PATHWAY	Neuroactive ligand-receptor interaction
805229	Glycogen synthase 1 (muscle)
KEGG PATHWAY	Starch and sucrose metabolism, insulin signaling pathway
PANTHER PATHWAY	P00026: Heterotrimeric G-protein signaling pathway-Gi alpha and Gs alpha mediated pathway
REACTOME PATHWAY	REACT_474: Metabolism of carbohydrates
814029	Growth differentiation factor 11
PANTHER PATHWAY	P00052: TGF-β signaling pathway
796746	Hairy and enhancer of split 7 (Drosophila)
BIOCARTA	Segmentation clock
805966	Heparan sulfate (glucosamine) 3-O-sulfotransferase 1
KEGG PATHWAY	Heparan sulfate biosynthesis
819555	Kalirin, RhoGEF kinase
PANTHER PATHWAY	P00029: Huntington's disease,
REACTOME PATHWAY	REACT_11044: Signaling by Rho GTPases
818352	Methionine adenosyltransferase II, alpha
KEGG PATHWAY	Cysteine and methionine metabolism, selenoamino acid metabolism,
PANTHER PATHWAY	P02773: S adenosyl methionine biosynthesis,
REACTOME PATHWAY	REACT_13433: Biological oxidations, REACT_13: Metabolism of amino acids
781573	Methylthioadenosine phosphorylase
KEGG PATHWAY	Cysteine and methionine metabolism
809388	Peripherin 2 (retinal degeneration, slow)
KEGG PATHWAY	Amyotrophic lateral sclerosis (ALS)
778054	Phospholipase C, beta 3 (phosphatidylinositol-specific)

KEGG PATHWAY	Inositol phosphate metabolism, calcium signaling pathway, chemokine signaling pathway, phosphatidylinositol signaling system, vascular smooth muscle contraction, Wnt signaling pathway, Gap junction, long-term potentiation, long-term depression, GnRH signaling pathway, melanogenesis, Alzheimer's disease, Huntington's disease
PANTHER PATHWAY	P00002: Alpha adrenergic receptor signaling pathway, P00019: Endothelin signaling pathway, P00027: Heterotrimeric G-protein signaling pathway-Gq alpha and Go alpha mediated pathway, P00031: Inflammation mediated by chemokine and cytokine signaling pathway, P00057: Wnt signaling pathway, P04374: 5HT2 type receptor mediated signaling pathway, P04385: Histamine H1 receptor mediated signaling pathway, P04391: Oxytocin receptor mediated signaling pathway, P04394: Thyrotropin-releasing hormone receptor signaling pathway, P05726: 2-arachidonoylglycerol biosynthesis, P05730: Endogenous_cannabinoid_signaling, P05911: Angiotensin II-stimulated signaling through G proteins and beta-arrestin
REACTOME PATHWAY	REACT_15295: Opioid Signaling, REACT_15380: Diabetes pathways, REACT_604: Hemostasis
808900	Poliovirus receptor-related 4
KEGG PATHWAY	Adherens junction
797522	Ras homolog gene family, member J
PANTHER PATHWAY	P00008: Axon guidance mediated by Slit/Robo, P00016: Cytoskeletal regulation by Rho GTPase, P00018: EGF receptor signaling pathway, P00029: Huntington disease
REACTOME PATHWAY	REACT_11044: Signaling by Rho GTPases
818356	Solute carrier family 43, member 1
REACTOME PATHWAY	REACT_13: Metabolism of amino acids, REACT_15518: Transmembrane transport of small molecules
818959	Solute carrier family 6 (neurotransmitter transporter, dopamine), member 3
KEGG PATHWAY	Parkinson's disease,
PANTHER PATHWAY	P00001: Adrenaline and noradrenaline biosynthesis, P00049: Parkinson’s disease, P05912: Dopamine receptor mediated signaling pathway
REACTOME PATHWAY	REACT_13685: Synaptic transmission
776944	t-SNARE domain containing 1
KEGG PATHWAY	SNARE interactions in vesicular transport
803123	Tankyrase, TRF1-interacting ankyrin-related ADP-ribose polymerase
BIOCARTA	Telomeres, telomerase, cellular aging, immortality
806191	V-ets erythroblastosis virus E26 oncogene homolog 1 (avian)
BIOCARTA	METS affect macrophage differentiation, keratinocyte differentiation
KEGG PATHWAY	Dorso-ventral axis formation, pathways in cancer, renal cell carcinoma
PANTHER PATHWAY	P00005: Angiogenesis, P00047:PDGF signaling pathway, P00056: VEGF signaling pathway, P04393:Ras pathway
787848	Wingless-type MMTV integration site family, member 9B
KEGG PATHWAY	Wnt signaling pathway, Hedgehog signaling pathway, melanogenesis, pathways in cancer, basal cell carcinoma
PANTHER PATHWAY	P00004: Alzheimer’s disease-presenilin pathway, P00012: Cadherin signaling pathway, P00057: Wnt signaling pathway
779082	Zinc finger, FYVE domain containing 9
BIOCARTA	TGF-β signaling pathway,
KEGG PATHWAY	TGF-β signaling pathway,
PANTHER PATHWAY	P00052:TGF-β signaling pathway,
REACTOME PATHWAY	REACT_6844: Signaling by TGF-β

and pluripotent capacity of a human adenocarcinoma gastric cell line.[24] In the present study, we aimed to investigate the effect of *PlGF* knockdown on hsa-miR-22-3p, hsa-let-7b-3p, hsa-miR-451b, and hsa-mir-4290 expressions in CSLCs derived from the MKN-45 cell line.

Recent studies have shown that hsa-mir-22 is a tumor suppressor miRNA in human gastric cancer.[25] Therefore, Su et al. have suggested that hsa-mir-22 acts as an oncomir in blood progenitor stem cells and targets the TET2 tumor suppressor gene.[26] Our study, in support of the previous studies, indicated that *PlGF* knockdown decreased expression of hsa-mir-22-3p in MKN-45-derived CSLCs. It demonstrated that overexpression of *PlGF* was likely connected with up-regulation of hsa-miR-22-3p in MKN-45-derived CSLCs and hsa-mir-22-3p acted as an oncomir. On the other hand, a recent study reported that *PlGF* increased AKT signaling pathway activity and showed the involvement of AKT in hsa-miR-22 overexpression.[27]

We used different bioinformatics tools to predict the putative targets and signaling pathways influenced by hsa-mir-22-3p. The bioinformatics results revealed that Fas (TNF receptor superfamily, member 6), a target gene of hsa-mir-22-3p, was a tumor suppressor gene that promoted apoptosis in the FAS and p53 pathways. hsa-mir-22-3p targeted cyclin G2 involved in the p53 signaling pathway.

Our results also indicated that *PlGF*-siRNA treatment up-regulated expression of hsa-let-7b-3p in CSLCs derived from MKN-45. In this regard, bioinformatics analysis showed that hsa-let-7b-3p acted as a tumor suppressor miRNA. Cyclin D2, as an oncogene, is a target of hsa-let-7b-3p and contributes to the cell cycle, PI3k, and Wnt signaling pathways. hsa-let-7b-3p targets endothelin 1 and inhibits the Wnt signaling pathway. However, hsa-let-7b-3p inhibits PDGF and VEGF signaling pathway by targeting MAPK6. *PlGF* plays an important role in up-regulation of c-MYC transcription factor and signaling pathways activated by c-MYC are involved in down-regulation of hsa-let-7b.[28-30]

Bioinformatics results also showed that target genes of hsa-mir-451b included *Sumo3*, *Tox3*, *Cacna1e*, *Cacnb4*, *Cdk2*, *Dner*, *Fnta*, and *Prkce*. *SUMO3* and *TOX3* are involved in the p53 signaling pathway. *CACNA1E* and *CACNB4* are involved in the MAPK signaling pathway. *CDK2* participates in cell cycle regulation and p53 signaling, whereas *DNER* is involved in Notch signaling pathway and the angiogenesis process. *FNTA* is involved in apoptosis and *PRKCE* has roles in angiogenesis, apoptosis, and the Wnt and VEGF signaling pathways. The experimental results of knock down of *PlGF* on the expression of hsa-mir-451b have confirmed our bioinformatics studies.

Bioinformatics results have also indicated that DnaJ (Hsp40), growth differentiation factor 11, phospholipase C-beta 3, v-ets erythroblastosis virus E26 oncogene homolog 1, and the wingless-type MMTV integration site family were target genes of hsa-mir-4290. DnaJ (Hsp40) plays important roles in hypoxia and the p53 signaling pathway. Growth differentiation factor 11 is involved in the TGF-beta signaling pathway. Phospholipase C-beta 3 is involved in the Wnt signaling pathway and v-ets erythroblastosis virus E26 oncogene homolog 1 participate in differentiation, angiogenesis, PDGF, VEGF, and Ras pathways. Finally, Wingless-type MMTV integration site family is involved in Wnt and the hedgehog signaling pathway. These results indicated that hsa-mir-451b and hsa-mir-4290 probably promote CSCs-related signaling pathways.

Conclusion

This study suggests that the *PlGF* signaling pathway influenced the expressions of hsa-miR-22-3p, hsa-let-7b-3p, hsa-miR-451b, and hsa-mir-4290 in MKN-45-derived CSLCs. Our bioinformatics analysis has proposed some component targets for these miRNAs. However, more experimental studies are necessary to identify the exact effects of *PlGF* knockdown on the targets of hsa-miR-22-3p, hsa-let-7b-3p, hsa-miR-451b, and hsa-mir-4290 in CSLCs derived from

MKN- 45 cells in an attempt to determine their genuine target genes.

Acknowledgements

We would like to acknowledge and extend our gratitude to Diba Farahani for her contribution in completing this work.

Funding sources

This work had no specific funding.

Authors' Contribution

Hassan Akrami designed and directed the investigation and wrote the manuscript. Zohreh Salehi contributed in CSLCs isolation and real-time PCR and Sajjad Sisakhtnezhad designed miRNA primers and contributed in real time PCR.

Conflict of interest

No conflict of interest is declared.

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