ORIGINAL RESEARCH

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PEG10 promotes the migration of human Burkitt's lymphoma cells by up-regulating the expression of matrix metalloproteinase-2 and -9

Abstract

Purpose: Paternally expressed gene 10 (*PEG10*) is important for apoptosis resistance in cancer cells; however, the effect of *PEG10* on tumor cell migration remains poorly understood. In this study, we investigated the effects of *PEG10* on proliferation, apoptosis, adhesion and migration in the Burkitt's lymphoma cell line, Raji.

Methods: Apoptosis was induced by 5-fluorouracil (5-FU) in pcDNA3.0/*PEG10* transiently transfected HEK293T cells and *PEG10*-suppressed Raji cells. siRNA*PEG10* was used to inhibit *PEG10* expression. Fluorescence-activated cell sorting (FACS) were performed to analyze the effect of *PEG10* on apoptosis. CCK-8 were performed to detect cell proliferation and adhesion. Matrigel invasion were performed using *PEG10*-suppressed Raji cells to investigate cell migration. The expression levels of matrix metalloproteinases -2 and -9 (*MMP-2* and *MMP-9*) were analyzed in *PEG10*-suppressed Raji cells using both real-time RT-PCR and Western blot analysis.

Results: HEK293T cells that overexpressed *PEG10* exhibited greater viability 48 h following treatment with 5-FU, relative to control cells. Specific inhibition of *PEG10* expression by siRNA resulted in inhibition of growth and apoptosis in Raji cells. Adherence and invasion capabilities were downregulated and expression levels of *MMP-2* and *MMP-9* were reduced in *PEG10*-suppressed Raji cells.

Conclusions: Our findings demonstrated that *PEG10* enhances the apoptotic resistance and viability of Raji cells. The migration and adherence invasion capacity of Raji cells could potentially be affected by regulation of the expression of *MMP-2* and *MMP-9*. Our research provides a promising strategy for cancer immunotherapy of lymphoma.

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Lymphoma is a malignant, proliferative hematopoietic cell disorder which leads to a variable survival time that ranges from months to decades. The incidence of Non-Hodgkin's lymphoma (NHL) has been rising continuously for some years and is now the fifth most common cancer. Burkitt's lymphoma (BL) is a unique neoplasm with the shortest doubling time and highest invasive potential. Its unequalled proliferation rate creates special challenges for diagnosis and treatment. The traditional therapeutic approaches for treatment of BL have been shown to be highly successful; however, recovery of patients with migration or recurrent disease remains very poor. Longterm survival is only 10-20% for migration or recurrent BL [1, 2].

Paternally expressed gene 10 (*PEG10*) is a gene from a newly defined imprinted region located at human chromosome 7q21 [3]. It has an elevated level of expression in the majority of the human hepatocellular carcinoma cells (HCC), and regenerating mouse liver cells [4, 5]. Exogenous expression of *PEG10* confers oncogenic activity and transfection of *PEG10* siRNAs to hepatoma cells and suppressing its expression results in cancer cell growth inhibition. Overexpression of *PEG10* decreases the cell death mediated by SIAH1, a mediator of apoptosis [6]. *PEG10* also appears to be important during placental development and during brain development [7-9].

Kainz reported elevated expression of *PEG10* in cases of high-risk B-cell chronic lymphocytic leukemia (B-CLL), which is defined by high lipoprotein lipase mRNA expression and long term inhibition of *PEG10* leading to induction of apoptosis in B-CLL cells [10]. In our previous research, B-cell acute lymphocytic leukemia (B-ALL) CD23+CD5+B cells the interaction of CCL19/CCR7 and CXCL13/CXCR5 were found to upregulate *PEG10* expression, which resulted in enhanced apoptotic resistance in these cells [11]. Other previously reported studies have demonstrated that, in addition to apoptosis, overexpressed levels of CCR7 and CXCR5 on tumor lymphocytes are closely related to cell migration and infiltration [12, 13]. The relationship between elevated expression of *PEG10* and the invasion and migration abilities of lymphoma cells remains unclear. We hypothesize that the elevated expression of *PEG10* not only enhances apoptotic resistance but also promotes the invasion and migration of lymphoma cells.

The hallmark of tumor cell invasion and migration is excessive degradation of the extracellular matrix. The matrix metalloproteinases (MMPs) are a multigene family of structurally related endopeptidases that are capable of collectively degrading all of the components of the extracellular matrix (ECM) [14]. MMPs have been implicated in many normal and disease-

related processes including development, arthritis and tumor invasion and migration [15, 16]. Among all of the MMPs, *MMP-2* and *MMP-9* have been shown to be critical enzymes in cancer cell invasion and migration. Overexpression of *MMP-2* and *MMP-9* is associated with increased dissemination and infiltration of lymphoma cells [17, 18].

To investigate whether *PEG10* plays a role in the apoptosis, migration and invasion of lymphoma cells, the effects of *PEG10* on proliferation, apoptosis, adhesion and invasion were investigated in Raji cells. *PEG10* is shown to enhance apoptotic resistance, proliferation and adherence. The invasion ability of Raji cells could potentially be affected by *PEG10* through regulation of the expression of *MMP-2* and *MMP-9*. Our findings demonstrate a potential new target for a novel therapeutic approach to treatment of lymphoma.

Materials and Methods

Cell culture and reagents

The human BL cell line, Raji, the human hepatoma cell line, BEL-7404, and HEK293T cells (Cell Bank of the Institute of Biochemistry and Cell Biology, SIBS, CAS) were grown in monolayers and cultured in RPMI1640 (Hyclone) or Dulbecco's modified Eagle's medium (DMEM) with High Glucose (Hyclone) and supplemented with 10% heat inactivated fetal bovine serum (Hyclone) and maintained at 37°C in 5% CO₂.

The open reading frame (ORF) of human *PEG10*, which is identical to NM_015068, contains 325 amino acids. Full-length cDNA corresponding to the ORF of *PEG10* was isolated from BEL-7404 cells, and then subcloned it into pcDNA3.0 plasmid between Hind III and EcoR I restriction sites. The orientation and correct frame of the recombinant vector pcDNA3.0/*PEG10* was confirmed by sequencing.

The *MMP-2/-9* monoclonal antibodies used for Western blot analysis were purchased from Biolegend. The secondary antibodies conjugated with peroxidase were purchased from Rockland. Other reagents included Cell Counting Kit-8 (Dojindo), Matrigel and Millicell plates (Millipore), Fibronectin (Chemicon), Oligofectamine and Opti-MEM (Invitrogen) and primers for *MMP-9/-2* and *GAPDH* (Invitrogen). SuperSignal West Pico Trial Kit Detection System (Pierce) was also utilized.

siRNA and transfection

SiRNA oligonucleotides were designed and synthesized as previously reported [19]. The siRNA against *PEG10* was named siRNAPEG10, and an unspecific control siRNA was composed of random sequences.

The sequences of siRNAs were as follows:

siRNAPEG10

Sense : 5' gucgugucugcugauuTT 3'

Antisense : 5' aaucagagcagacgcacTT 3'

Unspecific control siRNA

Sense : 5' ugaaggaguuccugauuTT 3'

Antisense : 5' agaagaucaggaacuccuuTT 3'

For the Oligofectamine transfection, 5×10⁵ cells were plated in 12-well plates prior to transfection in accordance with the manufacturer's instructions. Next, the siRNAs were diluted with an appropriate concentration of Opti-MEM I to form complexes. The cells were washed with RPMI1640 to remove serum and supplemented with 10% fetal calf serum 6 h post-transfection. The Raji cells were then transiently transfected with the complexes obtained by Oligofectamine. The HEK293T cells were transiently transfected with pcDNA3.0 and the recombinant vector pcDNA3.0/PEG10 by Lipofectamine 2000. The viability of the cells was tested by removing a portion of cells from the culture plate and counting them with a haemocytometer after trypan blue staining.

FACS analysis

For detection of apoptosis, cells were seeded on 6-well plates (5×10⁵ cells/well) and transfection were performed as described above. 5-Fluorouracil (5-FU) (100 µg/ml) was then added directly to the medium 24 h after transfection to induce apoptosis. After 72 h, cells were collected by trypsinization and washed twice with cold PBS. The cells were then fixed in citrate buffer and stained with propidium iodide (PI). The DNA content of the cells was estimated by fluorescence-activated cell sorting (FACS) analysis.

Cell proliferation assay

Raji cell suspension (100 µl, 5,000 cells/well) was dispensed in the 96-well plate at 37°C under 5% CO₂. The cells were transiently transfected with various concentrations of siRNAs by Oligofectamine. After incubation for 48 h, CCK-8 solution (10 µl) was added to each well of the plate. The plate was then incubated for 3 h. The absorbance was measured using a PerkinElmer 2030 VICTOR X Multilabel Plate Reader at 450 nm.

Cell adherence assay

Fibronectin (20 mg/L) was pre-incubated in the 96-well plate overnight at 37°C in 5% CO₂. Excess liquid was discarded and 100 µl 2% BSA was added to each well. After 1 h incubation, 100 µl of Raji cell suspension (5,000 cells/well) was dispensed into the 96-well plate. The cells were transiently transfected with various concentrations of siRNAs by Oligofectamine.

After incubation for 48 h, CCK-8 solution (10 µl) was added to each well of the plate. The plate was then incubated for 3 h. Finally, each plate was washed three times with PBS, aside from the control and one cell counting sample, and the absorbance rate was measured at 450 nm using a PerkinElmer 2030 VICTOR X Multilabel Plate Reader.

Real-time quantitative reverse transcription (RT)-PCR assay

The total RNA was extracted from cells using the Quick Prep total RNA extraction kit (Pharmacia Biotech) and digested by RNase free DNase at 37°C for 60 min. The DNase was then heat-inactivated at 65°C for 10 min. RNA was reverse transcribed using oligo (dT)18 and Reverse Transcriptase (Life Technologies). The samples were incubated at 42°C for 60 minutes, then heated at 95°C for 5 minutes to inactivate the Reverse Transcriptase. Real-time quantitative PCR was performed with ABI PRISMs 7700 SequenceDetector Systems. A SYBRs Green PCR Core Reagents Kit was used to generate fluorescence signals during each PCR cycle.

The sequences of the specific primers were as follows:

PEG10

Forward : 5'-AACAACAACAACAACCTCCAAGC-3'

Reverse : 5'-TCTGCACCTGGCTCTGCAG-3'

MMP-9

Forward : 5'-GAGGTTTCGACGTGAAGGCGCAGATG-3'

Reverse : 5'-CATAGGTCACGTAGCCCACTTGGTG-3'

MMP-2

Forward:5'-TGACATCAAGGGCATTTTCAGGAGC-3'

Reverse : 5'-GTCCGCCAAATGAACCGGTCCTTG-3'

GAPDH

Forward : 5'-ACAACAGCCTCAAGATCATCAG-3'

Reverse : 5'-GGTCCACCACTGACACGTTG-3'

The data were expressed as relative mRNA (gene) copies in 25 ng complementary DNA, which was normalized with the expression of a target gene to a housekeeping gene *GAPDH*. PCR reaction conditions were 2 min at 50°C, 10 min at 94°C, 40 cycles with 15 s at 94°C followed by 30 s at 60°C for amplifications.

Western blot assay

The polyclonal antibody to PEG10 was purified from sera of rabbits immunized with recombinant PEG10-his tag protein

produced in *E. coli*. Cell extracts were prepared using SDS loading buffer (4% SDS, 1% Triton X-100, 50 mM Tris-HCl, pH 7.4). Proteins were separated by 12% SDS-PAGE and transferred to PVDF membranes. The membranes were blocked with 5% non-fat milk T-TBS (0.1% Tween-20, 100 mM Tris-HCl, 0.9% NaCl) and incubated with antibodies. Goat peroxidase-conjugated affinity purified IgG served as the secondary antibody for the SuperSignal West Pico Trial Kit Detection System. Band intensity was measured by densitometry using the Quantity One software (Bio-Rad, Hercules, CA). *GAPDH* served as an internal control.

Matrigel invasion assay

Matrigel was thawed at 4°C overnight, and then diluted with serum-free cold RPMI1640. Raji cells were transfected with various concentrations of siRNAs using Oligofectamine. The cells were prepared for further treatment following incubation for 24 h. Briefly, the cell culture inserts (8 µm pore size) were coated with 100 µl Matrigel (2 mg/ml), placed in a 24-well plate, and incubated at 37°C to allow gelling. The gel was gently washed with warmed serum free- RPMI1640. Next, 100 µl of the transfected cell suspension was placed into the upper chamber, and 600 µl of serum free RPMI1640, containing 5 µg/ml fibronectin, was placed into the lower chamber of the transwell. The plate was incubated at 37°C for another 24 h. The transwell inserts were removed from the 24-well plates after incubation. Cells that had not migrated were scraped off with a cotton swab. The inserts were then fixed in methanol for 10 min at room temperature and stained with hematoxylin. Cells that had migrated to the lower surface were counted using a light microscope in five pre-determined fields at a magnification of 200 fold.

Statistical analysis

Experiments were performed in triplicate and repeated at least six times. Data are expressed as mean values $\pm$ standard deviation, and statistical significance was assessed by one-way ANOVA for independent groups using the SPSS 11.0 program. A value of $P < 0.05$ was considered to indicate a statistically significant difference.

Results

PEG10 plays an important role in apoptosis

A common chemotherapeutic agent, 5-FU is used in vitro as an inducer of apoptosis in cancer cells. In our study, a small dose

(100 µg/ml) of 5-FU was used to induce apoptosis in Raji cells. Our results demonstrated that the apoptosis rate of the blank control group was 2.93%, the 5-FU group was 15.47%, and the siRNA *PEG10* (40 nM) and unspecific control siRNA (40 nM) groups were 6.46% and 4.69%, respectively. Inhibition of *PEG10* expression alone did not affect the growth of Raji cells, when compared with the blank control, whereas siRNA *PEG10* and the unspecific control siRNA exhibited an effect when used along with 5-FU, with apoptosis rates of 30.83% and 16.80%, respectively (Fig 1). These results indicated that the specific inhibition of *PEG10* expression using siRNA *PEG10* enhanced the effect of 5-FU on apoptosis.

To further explore whether overexpression of *PEG10* promotes apoptosis resistance, the *PEG10* expression plasmid, pcDNA3.0/*PEG10*, was constructed. HEK293T cells were transfected with pcDNA3.0/*PEG10* or pcDNA3.0 at the same dose of 100 ng. Following 24 h of transfection, 5-FU was added to the culture medium, at a final dosage of 40 µg/ml, to induce apoptosis. The apoptosis rate of HEK293T cells was detected 72 h after transfection. The apoptosis rate of the 5-FU group was 15.14%. The apoptosis rates of the pcDNA3.0/*PEG10* and pcDNA3.0 groups were both less than 8%. Vector transfection alone did not affect the growth of HEK293T cells, whereas pcDNA3.0/*PEG10* and pcDNA3.0 worked in a complimentary manner with 5-FU; the apoptosis rates were 7.84% and 15.23%, respectively (Fig 2). HEK293T cells overexpressing *PEG10* exhibited a greater viability 48 h after treatment with 5-FU.

These results indicated that specific inhibition of *PEG10* expression could increase the apoptosis rate of Raji cells induced by 5-FU. In contrast, overexpression of *PEG10* would promote apoptosis resistance in Raji cells. This conclusion was consistent with our previous findings that *PEG10* plays a role in apoptosis in B-ALL cells [11].

PEG10 promotes cell proliferation

To determine if the proliferation characteristics changed after transfecting with siRNA *PEG10* in Raji cell, cell viability was evaluated by CCK-8. Raji cells were transfected with siRNA *PEG10L* (10 nM) and siRNA *PEG10H* (40 nM), respectively, and a control group was also transfected with unspecific siRNA. After the cells were incubated with different concentrations of siRNA *PEG10* for 48 h, the proliferation of *PEG10*-silencing cells was lower than control cells ($P_{low} < 0.05$, $P_{high} < 0.05$) (Fig. 3). This demonstrates that downregulation of *PEG10* expression, with both low (10 nM) and high (40 nM) concentrations of siRNA, significantly reduced the proliferation rate of Raji cells compared with the control. Prolifera-

FIGURE 1. Inhibition of *PEG10* expression enhanced the 5-FU-induced apoptosis of Raji cells. A combination of *PEG10* inhibition and 5-FU exposure increased apoptosis in Raji cells compared with either agent alone or the combination of unspecific siRNA and 5-FU.

FIGURE 2. Overexpression of *PEG10* increased resistance to apoptosis in HEK293T cells. Cells with overexpression of *PEG10* exhibited increased resistance to apoptosis induced by 5-FU compared with other groups.

FIGURE 3. *PEG10* affected the proliferation and adherence capabilities of Raji cells. Raji cells were analyzed following transfection with non-specific siRNA (40 nM), siRNAPEG10L (10 nM), and siRNAPEG10H (40 nM), respectively. The proliferation rate and adherence capabilities were reduced as the concentration of siRNAPEG10 increased. The data are presented as mean value $\pm$ SD of six experiments in each group. (* $P < 0.05$ compared to control, one-way ANOVA)

tion rate was more effectively suppressed in cells transfected with the high concentration of siRNA compared with the low concentration ($P < 0.05$). Our findings indicated that a high concentration of siRNA was more efficient in abolishing *PEG10* expression in Raji cells (Fig 5) and that the expression level of *PEG10* was closely related to the proliferation of Raji cells in a dose-dependent manner.

PEG10 enhances cell adherence ability

We next examined the effect of *PEG10* on cell adhesion. The results indicated that downregulation of *PEG10* expression with both low (10 nM) and high (40 nM) concentrations of siRNA significantly reduced the adherence capability of Raji cells compared to the control ($P_{low} < 0.05$, $P_{high} < 0.05$, Fig 3). *PEG10* affected the adherence capability in a dose-dependent manner.

PEG10 is closely related to the expression level of MMP-2 and MMP-9

Among the proteinases capable of degrading the ECM, the matrix metalloproteinases (*MMP-2* and *MMP-9*) appear to be particularly important [17, 18]. To explore the effect of *PEG10* on the invasion and migration capabilities of Raji cells, the mRNA and protein expression levels of these proteinases in *PEG10*-suppressed Raji cells were analysed. The results of real-

time RT-PCR and Western blot analysis indicated that the expression levels of *MMP-2* and *MMP-9* were inhibited 48 hours after abolishing the expression of *PEG10*. As the concentration of siRNAPEG10 increased, *PEG10* expression was effectively suppressed, and both the *MMP-2* and *MMP-9* mRNA and protein expression levels gradually declined (Fig 4), in a manner similar to the results of the adherence assay that we presented. These results indicate that the expression level of *PEG10* is closely related to the expression level of *MMP-2* and *MMP-9*.

PEG10 promotes the migration and invasion abilities

Matrigel invasion assays were performed to further assess the effect of *PEG10* on the invasion potential of Raji cells (Fig 5). In the control group, the Raji cells were transfected with non-specific siRNA. The cells in the control group migrated through the Matrigel freely. When Raji cells were transfected with siRNAPEG10, the migration ability was significantly reduced ($P < 0.05$). Quantitative analysis demonstrated that the number of Raji cells migrating through the Matrigel was less in the group in which cells were transfected with high concentrations of siRNAPEG10 compared with low concentrations of siRNAPEG10 ($P < 0.05$). This finding provided strong evidence that, with downregulation of *PEG10* expression, the migration and invasion abilities of Raji cells were significantly inhibited compared with controls, and that this downregulation occurs

FIGURE 4. Expression levels of MMP-9 and MMP-2. The mRNA (A) and protein (C) expression levels of PEG10, MMP-2, MMP-9, and GAPDH of Raji cells were analyzed after transfection with siRNAPEG10L (10 nM) and siRNAPEG10H (40 nM), respectively. Bars represent the relative mRNA copies or optical density (OD) values normalized to GAPDH (real-time RT-PCR, figure B; Western blot, figure D, respectively). The data of B and D are expressed as mean value $\pm$ SD from at least six independent experiments.

FIGURE 5. Effect of *PEG10* on migration and invasion abilities of Raji cells. Invasion ability of Raji cells was measured after transfection with control (nonspecific siRNA, 40 nM), siRNAPEG10L (10 nM), and siRNAPEG10H (40 nM), respectively. The invasion capabilities were reduced as the concentration of siRNAPEG10 increased. The data were mean value $\pm$ SD of six experiments in each group. (* $P < 0.05$ compared to control, one-way ANOVA)

in a dose-dependent manner. These results indicate that *PEG10* is critical for the migration and invasion capabilities of Raji cells.

Discussion

BL is an aggressive B-cell neoplasm and is one of the most common lymphomas, especially in childhood. Although poly-chemotherapy regimens have improved clinical outcomes and changed the prognoses for BL patients, the high invasion and recurrence are still the most difficult aspects of the treatment.

PEG10 is identified as a maternally-imprinted and paternally expressed gene located at human chromosome 7q21 that functions as a transcriptional factor [3, 7]. Our previous reports have demonstrated that B-ALL *CCL19/CCR7* and *CXCL13/CXCR5* together upregulate *PEG10* expression, and result in the enhancement of the apoptotic resistance of B-ALL CD23+CD5+B cells [11]. In this study, the role of *PEG10* in lymphoma cell proliferation, apoptosis resistance, adhesion, migration and invasion was investigated.

The inhibition of *PEG10* expression enhanced the apoptosis induced by 5-FU in Raji cells, whereas overexpression of *PEG10* promoted the resistance to apoptosis induced by 5-FU. These results were consistent with our previous findings that upregulation of *PEG10* expression enhances the apoptotic resistance of leukemic cells [11]. Our findings also suggest that *PEG10* functions as an important oncogene in the progression of lymphoma by inhibiting cell growth and increasing tolerance to apoptosis. This has already been demonstrated in HCC cell lines and may involve interaction with *SLAH1* [6].

Tumor cell invasion is a multistep phenomenon, in which extensive degradation of the matrix is the most critical step [20]. *MMP-2* and *MMP-9* are enzymes that degrade collagen type IV, the principal component of basement membranes, and are thought to be associated with the transmigration and degradation of the ECM structures of tissue and blood vessels [14, 21]. In this study, the expression levels of *MMP-2* and *MMP-9* were significantly inhibited, both at the mRNA and protein levels, through the downregulation of *PEG10* expression. Since *MMP-2* and *MMP-9* are closely related to the invasion phenotype of cancer cells, our results further suggest that *PEG10* expression levels affect the invasion capabilities of cancer cells. This deduction was demonstrated in the Matrigel invasion assays: the invasion capabilities of Raji cells were significantly reduced as *PEG10* expression was suppressed. In addition, the cell adhesion abilities of Raji cells were also decreased upon downregulation of *PEG10* expression.

In this study *PEG10* was found to enhance the adhesion, migration and invasion capabilities of Raji cells. The excessive

egress of cells from the marrow into peripheral blood and subsequent infiltration into various tissues is crucial for the malignant progression [22]. In order for these cells to invade and migrate to new sites in the body, they must first attain the ability to catalytically modify the ECM and the basement membranes and *PEG10* may promote the progression of these crucial steps. Thus, *PEG10* activity is important for the prognosis of BL: by downregulating *PEG10* expression, the malignant progression of BL could potentially be suppressed.

In summary, our study demonstrates the importance of *PEG10* expression in BL cell apoptosis resistance, proliferation, adhesion, migration and invasion. *PEG10* is potentially useful as a prognostic marker for the aggressiveness of BL. Our results add to the understanding of the role of *PEG10*, and provide a new molecular target for blocking BL invasion. Further investigation into *PEG10* expression in additional lymphoma cell lines and clinical samples is warranted.

Acknowledgments

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