Inhibition of Pim–1 attenuates the proliferation and migration in nasopharyngeal carcinoma cells

Wei Jie1, Qi-Yi He2, Bo-Tao Luo1, Shao-Jiang Zheng2, Yue-Qiong Kong2, Han–Guo Jiang1, Ru–Jia Li1, Jun–Li Guo2*, Zhi-Hua Shen1*

1Department of Pathology & Pathophysiology, School of Basic Medicine Science, Guangdong Medical College, Zhanjiang 524023, PR China
2Hainan Provincial Key Laboratory of Tropical Medicine, Hainan Medical College, Haikou 571199, PR China

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Abstract

Objective: To explore the role of proto–oncogene Pim–1 in the proliferation and migration of nasopharyngeal carcinoma (NPC) cells. Methods: Pim–1 expressions in NPC cell lines CNE1, CNE1–GL, CNE–2Z and C666–1 were examined by RT–PCR, western blotting and immunofluorescence, respectively. After CNE1, CNE1–GL and C666–1 cells were treated with different concentrations of Pim–1 special inhibitor, quercetagetin, the cell viability, colony formation rate and migration ability were analyzed. Results: Pim–1 expression was negative in well–differentiated CNE1 cells, whereas expressed weakly positive in poor–differentiated CNE–2Z cells and strongly positive in undifferentiated C666–1 cells. Interestingly, CNE1–GL cells that derived from CNE1 transfected with an Epstein Barr virus latent membrane protein–1 over–expression plasmid displayed stronger expression of Pim–1. Treatment of CNE1–GL and C666–1 cells with quercetagetin significantly decreased the cell viability, colony formation rate and migration ability but not the CNE1 cells. Conclusions: These findings suggest that Pim–1 overexpression contributes to NPC proliferation and migration, and targeting Pim–1 may be a potential treatment for anti–Pim–1–expressed NPCs.

1. Introduction

Nasopharyngeal carcinoma (NPC) is one of the most common head and neck carcinomas in Southern China, special in Guangdong, Hainan, Guangxi, Hunan and Fujian provinces[1]. Genetic factors and environmental factors including Epstein–Barr virus are the two important risk factors for NPC. Although the advanced equipments and technologies in radiotherapy and chemotherapy have been greatly applied in recent years, the 5–year survival rate of patients suffered with NPC remains no more than 70%.

Provirus integration site for Moloney murine leukemia virus 1 (Pim–1) is one of the Serine/threonine kinases, which is firstly reported in hematological malignancies[2]. Due to the ability of co–operation with c–Myc and Gfi1, Pim–1 is also deemed as a proto–oncogene and crucial player in the process of malignant transformation[3–4], however, its role in nasopharyngeal carcinoma remains unclear.

In this investigation, we examined the expression level of Pim–1 and studied its roles in various differentiated cell lines of NPC. Our results showed that the up–regulation of Pim–1 was associated with the differentiation type of NPC and administration of Pim–1 special inhibitor, quercetagetin, effectively suppressed NPC cells proliferation and migration. Our results indicate that over–expression of Pim–1 contributes to NPC progression, and targeting Pim–1 may be a potential interest strategy in NPC treatments.
2. Materials and methods

2.1. Cell culture

NPC cell lines CNE1 (well-differentiated, obtained from Institute of Virology, Chinese Academy of Preventive Medicine), CNE–2Z (poor-differentiated, established in our lab in 1980s) and C666–1 (undifferentiated, obtained from Sun Yat–Sen University Cancer Center) were grown in Dulbecco’s modified Eagle’s medium (DMEM, Hyclone, China) supplemented with 10% fetal bovine serum (FBS, Hyclone, China) and 100 U/mL penicillin and 0.1 mg/mL streptomycin. NPC cell line CNE1–GL was derived from CNE1 cells transfected with EBV LMP–1 eukaryotic expression plasmid PAT–GFP–LMP1, which was established previously in our lab, was maintained in DMEM contained with 10% FBS, 0.5 μg/mL puromycin (Signal–Aldrich, Shanghai, China), 100 U/mL penicillin and 0.1 mg/mL streptomycin. All cells were maintained at 37℃ in a 5% CO₂ atmosphere.

2.2. Drug treatment and cell viability

Inhibitor of Pim–1, quercetagetin (Cal Chemical Corp, Coventry, RI, USA), was dissolved in dimethyl sulfoxide (DMSO), and the final concentration of DMSO in completed medium was 0.3% (v/v). CNE1, CNE1–GL, CNE–2Z and C666–1 cells (2 × 10³/ well) were placed in 96–well plates. Cells were cultured in DMEM supplemented with quercetagetin (0, 0.55, 5.5, 11 μM) or DMSO (0.3%, v/v) for 48 hours. After twice washed with PBS, total 10 μL DMEM plus 10 μL Cell Counting Kit–8 (CCK–8) reagents (Biyuntian, Jiangsu, China) were added per wells for additional 2 hours, and then the optical density (OD) was measured using a microplate reader (Multiskan MKS, Thermo Scientific, Waltham, MA, USA).

2.3. Colony formation assay

CNE1, CNE1–GL and C666–1 cells treated with or without Pim–1 special inhibitor quercetagetin (5.5 μM) were seeded in 6–well plates (500 cells/well) and maintained in complete medium for up to two weeks. The cell colonies were then visualized by Giemsa staining.

2.4. RNA extraction and RT–PCR

Total RNA was extracted with TaKaRa RNAiso plus reagent (TaKaRa Biotechnology (Dalian) Co., Ltd.). The procedure of RT–PCR was performed according to the instruction of PrimeScript® One Step RT–PCR Kit Ver.2 (TaKaRa). 1 μg total RNA was used as a template and cDNAs was amplified by Mastercycler® Gradient Thermal cycler (Eppendorf, Germany) at the annealing temperature of 60 ℃ with total 29 cycles. The pairs of primers synthesized by Sangon Biotech Co., Ltd.(Shanghai, China) as follows were used: Pim–1 forward: 5’–GCT ACG ATG GGA CCC GAG TGT–3’, Pim–1 reverse: 5’–GAT TCT CTA TGG GAG GAG TTG AGG C–3’, yielding a 575 bp product; β–actin forward: 5’–GCT GGA CAT CGGCAA AGA C–3’, β–actin reverse: 5’–AAG AAA GGG TGT AAC GCA ACT–3’, yielding a 306 bp product. Products of PCR were separated by 1.5% agarose gel electrophoresis and visualized under UV using InGenius LHR gel Documentation and analysis system (InGene). The transcription levels of β–actin served as a loading control.

2.5. Western blotting

Western blotting was performed as described previously[5–7]. Protein homogenates were separated by SDS–PAGE using gel electrophoresis and transferred to a nitrocellulose membrane (BioRad, Hercules, CA, USA). Membranes were incubated with primary antibody against Pim–1 (1:300, sc–7859, Santa Cruz Biotechnology, Santa Cruz, CA, USA), p–BAD (Ser112) (1:1 000, Cell Signaling, MA, USA) and BAD (1:1 000, Cell Signaling, MA, USA) in TBST plus 5% skimmed milk overnight at 4 ℃. Membranes were washed and incubated with HRP–conjugated secondary antibodies for 1 hour at room temperature. Bands were visualized using enhanced chemiluminescent reagents (Thermo Fisher, Rockford, IL, USA) and analyzed using an InGenius LHR gel Documentation and analysis system (InGene, Fredrick, MO, USA).

2.6. Immunofluorescent staining

Indirect immunofluorescence was performed on NPC cells grown on glass coverlips as described previously[8]. After incubation overnight with the primary antibodies (Pim–1, 1:100) at 4 ℃, the antigenic sites were localized using FITC–conjugated rabbit anti–goat IgG (1:100, Protein Tech Group, Inc, Chicago, IL, USA). Images of the antigenic sites were captured by a laser scanning confocal microscope (TCS SP5 II; Leica, Germany).

2.7. Transwell migration assay

Transwell migration assay was performed as described previously[5,9]. Briefly, 8 μm pore size polycarbonate membranes (BD Biosciences, NJ, USA) were coated with Matrigel (100 μg/cm²; BD Biosciences) and incubated overnight. CNE1, CNE1–GL and C666–1 cells were treated with quercetagetin (0, 5.5 μM) or DMSO (0.3%, v/v) for 48 hours, then 2 × 10⁵ cells in DMEM supplemented with 1% BSA together with quercetagetin (0, 5.5 μM) or DMSO (0.3%, v/v) were placed in upper chamber. The bottom chamber consisted of medium DMEM with 10% FBS was used as a chemoattractant. Cells were incubated for additional 6 hours, after which the inserted membranes were fixed for visualization by Giemsa staining.
20 minutes in 4% paraformaldehyde in PBS and stained with Eosin staining. Then membranes were washed with PBS and cut from the inserts. Cells on the upper surface of the membrane were scraped with cotton swab and membranes were mounted with glycerol. Invaded cells on the lower surfaces of the membranes were counted within ten representative fields in triplicate inserts.

2.8. Statistical analysis

The statistical analysis was carried out by using PRISM Software (GraphPad Software, CA, USA). The data were expressed as Mean±SD. For analysis of differences between two groups, Student’s t-test was performed. For multiple groups, ANOVA was carried out followed by Student–Newman–Keuls test. The level of statistical significance was set at P<0.05.

3. Results

3.1. Expression profile of Pim-1 in NPC cell lines

To examine the expression profile of Pim-1 in different NPC cell lines, CNE1, CNE1-GL, CNE-2Z and C666-1 cells were used in the present study. As shown in Figure 1, a variable expression of Pim-1 was observed both at mRNA and protein levels in NPC cell lines. In general, a negative expression of Pim-1 was detected in well-differentiated CNE1 cells, while an increased expression of Pim-1 was observed in CNE-2Z cells (poor-differentiated NPC) and C666-1 cells (undifferentiated NPC). To our interest, the CNE1-GL cells that transfected with an EBV-LMP1 over-expression plasmid displayed the stronger Pim-1 expression, and C666-1 cells that EBV stably harbored while LMP-1 expressed very low levels [10], also displayed a moderate Pim-1 expression. Finally, results of immunofluorescent staining showed that Pim-1 was located in cytoplasm and nuclear in different NPC cell lines (Figure 2), which was similar to the expression profile of Pim-1 in some solid tumors [11-13].

3.2. Administration of quercetagetin attenuates NPC proliferation

We further examined the effects of targeting Pim-1 on changes of cell phenotypes. Pim-1 kinase has showed a promotion to cells proliferation. Thus, we examined the role of Pim-1 in NPC cells proliferation. As shown by Figure 3, administration of quercetagetin, a selected Pim-1 inhibitor [14,15], lead to decrease of cell viability (Figure 3a) and colony formation ability (Figure 3b) in the Pim-1-expressed CNE1-GL and C666-1 cells but not the Pim-1 negative CNE1 cells, suggesting Pim-1 expression contributes to NPC cells proliferation.

Figure 1. Pim-1 expression in different NPC cell lines. CNE1, CNE1-GL, CNE2Z and C666-1 cells were seeded in 24-well plates, 3 wells for each cell lines (n = 3) were harvested for the analysis of Pim-1 Expression by RT-PCR (a) and western blotting analysis (b). * P<0.05 vs. CNE1, # P<0.05 vs. CNE1-GL.

Figure 2. Expression and cellular location of Pim-1 in NPC cell lines examined by immunofluorescence. NPC cells were grown on glass coverlips, after incubated the coverlips with primary antibody overnight, the antigenic sites of Pim-1 were located by TRITC-conjugated rabbit-anti goat IgGs and observed by confocal microscope. Scale bar = 50 μM.

Figure 3. Effects of administration of quercetagetin on NPC cells proliferation. After the CNE1-GL, C666-1 and CNE1 were treated with different concentrations of Quercetagetin or DMSO (0.3%,v/v), the cell viability (a), colony formation rates (b) were assessed, respectively. * P<0.05 vs. DMSO or control group (Quercetagetin 0 μM), # P<0.05 vs. DMSO or control group (Quercetagetin 0 μM).
3.3. Effect of quercetagetin on NPC migration

The results of transwell assay showed that when quercetagetin was added, a decreased number of invaded NPC cells passed through the 8–μM pore sized polycarbonate membrane were observed in CNE1–GL and C666–1 cells (Figure 4), indicating inhibition of Pim–1 activity repressed NPC cells migration.

![Figure 4](image)

Figure 4. Effects of administration of quercetagetin on NPC cells migration.

After the CNE1–GL, C666–1 and CNE1 were treated with quercetagetin (0, 5.5 μM) or DMSO (0.3%, v/v), the migratory ability was assessed. Invaded cells on the lower surfaces of the membranes were counted within ten representative fields in triplicate inserts. * P<0.05 vs. DMSO or control group (Quercetagetin 0 μM), # P<0.05 vs. DMSO or control group (Quercetagetin 0 μM). Original magnification: ×100.

3.4. Quercetagetin decreases Pim–1 activity in NPC cells

Administration of quercetagetin showed no significant influence on Pim–1 expression (Figure 5a), thus, the Pim–1 activity was further assessed. Pim–1 kinase promotes inactivation of the BAD protein by phosphorylating it on the Ser112[16,17], thus, the modulation of the level of p–BAD at Ser112 implies the change of Pim–1 kinase activity. As shown in Figure 5b, treatment with quercetagetin significantly attenuated the levels of endogenous p–BAD (Ser112) in CNE1–GL cells but not in CNE1 cells, indicating administration of quercetagetin decreases Pim–1 activity in Pim–1–expressed NPC cells.

![Figure 5](image)

Figure 5. Effects of quercetagetin on Pim–1 expression and activity.

a Pim–1 expression at transcription and translation levels was assessed by RT–PCR and western blotting in CNE1–GL and C666–1 cells. b Changes of p–BAD (Ser112) and BAD were examined by western blotting in CNE1–GL and CNE1 cells.

4. Discussion

The present study firstly analyzed Pim–1 expression in different types of NPC cell lines. Generally, well–differentiated NPC cells CNE1 displayed a negative Pim–1 expression, however, poor–differentiated CNE2Z and undifferentiated C666–1 cells exhibited a much stronger Pim–1 expression. These results indicated that Pim–1 may be closely related to the NPC progression. The results of this studies also demonstrated that the expression of Pim–1 play a pivotal role in the proliferation and migration of NPC cells; when administration of quercetagetin, a selected Pim–1 activity inhibitor[14], significantly attenuated NPC proliferation and migration, suggesting that targeting Pim–1 was a potential promising therapeutic approach for NPC.

Pim–1 contributes to the cell proliferation due to the cell cycle progression and decrease of apoptosis[18]. Morishita et al founded that Pim–1 kinases were phosphorylated
and then down-regulated p27Kip1 at the transcriptional and posttranscriptional levels[19]. Cde25A/C, a direct transcriptional target for c-Myc, was physically interacted with and phosphorylated by Pim–1[20]. Besides, Pim–1 promoted complex formation between NuMA, HP1beta, dynein and dynactin, a complex that is necessary for mitosis. p100, a transcriptional co-activator that interacts with the c–Myb transcription factor, was bind and phosphorylated by Pim–1, too[21]. BAD is a proapoptotic member of the Bcl–2 family that promotes cell death by displacing Bax from binding to Bcl–2 and Bcl–xL. Phosphorylation of BAD at Ser112 and Ser136 promotes binding of BAD to 14–3–3 proteins to prevent an association between BAD with Bcl–2 and Bcl–xL[22]. It has been shown that Pim–1 may play a pivotal role in the regulation of the survival signaling via modulation of Bcl–2 family member like BAX[18,17,23]. Pim–1 kinase inactivates the BAD protein by phosphorylating it on the Ser112[16,17], thus, the modulation of the level of p–BAD at Ser112 implies the change of Pim–1 kinase activity. In the present study, administration of Pim–1 inhibitor, quercetagetin, showed no any significant impacts on Pim–1 expression; however, a decreased p–BAD (Ser112) levels were revealed in Pim–1–expressed CNE1–GL and C666–1 cells, suggesting administration of quercetagetin represses Pim–1 activity in NPC cells. In turn, a decrease of colony formation rate and cell viability was observed. Our results consisted with others reports in that treatment with Pim–1 inhibitors effectively induced a cell cycle arrest[24–27], and administration of Pim–1 siRNA inhibited cell proliferation[11,28–30].

It is well known that there is a close relationship between NPC and EBV infection, especially the poor– and undifferentiated NPC histological types. Latent membrane protein–1 (LMP–1) is EBV–encoded oncoprotein that could promote NPC to progress via activation of multiple signaling[31,32]. Recently, Kim et al reported that LMP–1 could increase chemo–resistance via cytoplasmic sequestration of Pim–1 in MCF7 and Jurkat cells[33], suggesting that LMP–1–induced Pim–1 expression in lymphomas. In the present study, the well–differentiated NPC cell line CNE1 showed no Pim–1 expression, however, the CNE1–GL that established from CNE1 transfected with a LMP–1 over–expression plasmid[34], displayed a very strong Pim–1 expression. However, the C666–1 cells that EBV consistently harbored demonstrated a very low level of LMP–1[10] at the same time, showed a moderate Pim–1 expression. The results of immunofluorescence revealed that Pim–1 located both in the nuclear and cytoplasm of NPC cells, however, an increase of Pim–1 expression in nuclear of CNE1–GL than C666–1 cells was evidenced by western blotting analysis (data not shown). These results indicated that LMP–1 could induce Pim–1 expression and may shift its cellular location. Based on the above results, it may be achieved that EBV infection could induced Pim–1 expression in NPC cells at least partially via LMP–1–activated singling. Further studies on the underlying mechanisms of EBV–induced Pim–1 expression and shift of cellular location are under investigation.

To the best of our knowledge, it is the first report regarding the relationship between Pim–1 and NPCs. In the present study, we found that over–expression of Pim–1 was associated with NPC cells proliferation and migration; moreover, administration of Pim–1 special inhibitor, quercetagetin, effectively attenuated Pim–1 activity in NPC cells, which lead to a decreased cell proliferation and migration. In sum, our study provides evidences that targeting Pim–1 would be a potential promising approach in Pim–1–expressed NPCs.

Conflict of interest statement

We declare that we have no conflict of interest.

References


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