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Differential regulation of cyclin-dependent kinase inhibitors in neuroblastoma cells

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ABSTRACT

Gastrin-releasing peptide (GRP) and its receptor (GRP-R) are highly expressed in undifferentiated neuroblastoma, and they play critical roles in oncogenesis. We previously reported that GRP activates the PI3K/AKT signaling pathway to promote DNA synthesis and cell cycle progression in neuroblastoma cells. Conversely, GRP-R silencing induces cell cycle arrest. Here, we speculated that GRP/GRP-R signaling induces neuroblastoma cell proliferation via regulation of cyclin-dependent kinase (CDK) inhibitors. Surprisingly, we found that GRP/GRP-R differentially induced expressions of p21 and p27. Silencing GRP/GRP-R decreased p21, but it increased p27 expressions in neuroblastoma cells. Furthermore, we found that the intracellular localization of p21 and p27 in the nuclear and cytoplasmic compartments, respectively. In addition, we found that GRP/GRP-R silencing increased the expression and accumulation of PTEN in the cytoplasm of neuroblastoma cells where it co-localized with p27, thus suggesting that p27 promotes the function of PTEN as a tumor suppressor by stabilizing PTEN in the cytoplasm. GRP/GRP-R regulation of CDK inhibitors and tumor suppressor PTEN may be critical for tumoriogenesis of neuroblastoma.

1. Introduction

As a neuroendocrine tumor, neuroblastoma can produce and secrete peptides, such as gastrin-releasing peptide (GRP), that alter cell functions [1]. GRP binds to gastrin-releasing peptide receptors (GRP-R) to activate various cellular processes for malignant transformation of neuroblastoma cells [2]. Phosphatidylinositol 3-kinase (PI3K) is required for diverse cellular activities, most notably in cellular proliferation and survival [3]. PTEN (Phosphatase and tensin homologue deleted on chromosome 10) is a tumor suppressor with a dual phosphatase function for lipid and protein, and is known as an endogenous PI3K inhibitor. Increased ratio of PI3K to PTEN has been found in various cancers [4].

We have previously shown that GRP/GRP-R signaling activates the PI3K/AKT signaling pathway, and the expression of PTEN correlates to cellular differentiation [1]. Modulated expression of GRP-R negatively regulated PTEN expression in neuroblastoma cells [1,2]. GRP-R overexpression has been shown to stimulate cell proliferation, DNA synthesis, and cell cycle progression in neuroblastoma [2]. We reported that GRP stimulates neuroblastoma cell growth by regulating the cell cycle [5]. Similarly, GRP-R silencing induced cell cycle arrest at G2/M phase in BE(2)-C cells [2], indicating that GRP/GRP-R act as important mediators of neuroblastoma tumorigenesis.

Cyclin-dependent kinase (CDK) inhibitors, p21 and p27, belong to the Cip/Kip family of CDK inhibitors and inhibit proliferation mainly by negatively regulating the cell cycle. The p21 protein not only acts as an assembly factor for CDK4, CDK6, and cyclin D-type complexes that initiate entry into the S phase of the cell cycle [6,7], but p21 also is a CDK2 inhibitor that initiates G1 arrest in response to DNA damage [8]. In neuroblastoma, p21 functions as p53-independent tumor suppressor [9]. p27 inhibitory domain binds to CDK2/cyclin E and CDK2/cyclin A complexes in the nucleus arresting cells at G1/S in the cell cycle. p27 is necessary for coordinating cell cycle and cell-death programs so that cell viability is maintained during exit from the cell cycle [10]. Overexpression of p27 has been shown to inhibit human neuroblastoma cell growth [11]. p27 inhibited the activity of CDKs during differentiation of N2A neuroblastoma cells [12]. Both p21 and p27 are differentially regulated by PI3K/PTEN signaling pathway. p21 can be phosphorylated by AKT1 directly, and therefore, disrupt the association with PCNA, and decreases CDK2 inhibition and promotes endothelial cell proliferation [13]. p27 is a critical mediator for the signaling pathway regulated by the PTEN tumor suppressor,

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because the loss of PTEN affects cell cycle progression by downregulation of p27 at its protein level [14]. However, the molecular mechanism involved in PI3K/PTEN mediated regulation of GRP/GRP-R-induced cell cycle progression is still unknown.

In this study, we investigated the molecular mechanisms involved in GRP/GRP-R-mediated regulation of CDK inhibitors, and determined the role of PTEN on cell cycle inhibitors, especially p27. Our findings demonstrate that inhibition of GRP or GRP-R led to coordinated increases in PTEN and p27 expressions, however, p21 was downregulated.

2. Materials and methods

2.1. Materials

Antibodies against PTEN, p21, Lamin A/C and 10× cell lysis buffer were purchased from Cell Signaling (Beverly, MA). Antibody against p27 was purchased from BD Transduction Laboratories (San Jose, CA). Anti β-actin and α-tubulin antibodies and fetal bovine serum (FBS) were from Sigma (St. Louis, MO). Alexa Fluor 568 goat anti-mouse and anti-rabbit secondary antibodies were from Life Technologies (Grand Island, NY). Horseradish peroxidase (HRP)-conjugated secondary antibodies against mouse and rabbit IgG were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Enhanced chemiluminescence (ECL) HRP substrate was purchased from EMD Millipore (Billerica, MA) and Perkin Elmer (Hebron, KY).

2.2. Cell culture and transfection

Human neuroblastoma cell line, BE(2)-C, was purchased from American Type Culture Collection (Manassas, VA). Cells were cultured in RPMI 1640 media (Cellgro Mediatech Inc., Herndon, VA) supplemented with 10% FBS in a humidified atmosphere of 5% CO2 at 37 °C. Stably transfected cells BE(2)-C/shCON and BE(2)-C/shGRP-R were established and cultured in media containing Zeocin (50 μg/ml) as formerly described [2]. Inducible GRP silencing system BE(2)-C/Tet/shGRP was cultured under selection media with blasticidin at 8 μg/ml and zeocin at 50 μg/ml as described in our previous report [15].

2.3. Western blot analysis

Proteins were quantified using the Bio-Rad Protein Assay kit (Hercules, CA). Equal amounts of protein were fractionated by electrophoresis on 4–12% NuPAGE Novex Bis-Tris gels (Invitrogen, Grand Island, NY), transferred to PVDF membranes, and probed with antibodies. The bands were visualized by an enhanced chemiluminescent detection system according to the manufacturer's instructions (Amersham Inc., Piscataway, NJ).

2.4. Cell fractionation

Cells were grown to 95% confluence, harvested by centrifugation at 200*g* for 5 min after trypsinzation. Cell pellet was resuspended in hypotonic buffer (100 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl and protease inhibitors) on ice for 20 min. Cells were lysed using a 25-gauge needle. Cells were centrifuged for 10 min at 400*g*, supernatant was collected as the cytoplasmic fraction. The pellet was further washed with hypotonic buffer and resuspended in cell lysis buffer. After sonication, the lysate was centrifuged for 10 min to obtain the nuclear fraction. β-tubulin and Lamin A/C were used as markers for cytoplasmic and nuclear fractions, respectively.

2.5. Immunofluorescent staining

Cells were washed once with phosphate buffered saline (PBS) and fixed in 4% paraformaldehyde in PBS for 15 min at room temperature (RT), then incubated in ice cold 100% methanol for 10 min at RT. Fixed cells were rehydrated in PBS for 30 min at RT, then blocked in 1% BSA/PBS buffer for 30 min, and incubated for 60 min in 1:100 dilution of anti-p21, or p27, or PTEN antibodies. Cells were washed three times with PBS and incubated 30 min in PBS containing 1% BSA and 1:500 secondary antibody Alexa Flour 568 goat anti-rabbit. Cells were rinsed three times with PBS, and coverslips were mounted onto the slide glasses with 1 drop of mounting medium with DAP1 (Vector Laboratories. Burlingame, CA). Images were obtained using a fluorescent microscope (Nikon Eclipse E600).

2.6. Statistical analysis

Scoring index, relative expression values were expressed as means ± SEM; statistical analyses were performed using student t-test for comparisons between the treatment groups. A p value of <0.05 was considered significant.

3. Results

3.1. GRP-R silencing decreased p21 expression

The PI3K/AKT pathway promotes cell proliferation by modulating cell cycle progression, including the nuclear accumulation of cyclin D, which induces G1/S phase progression [16,17]. The PI3K can also regulate tumor suppressors p21 and p27, the negative regulators of the cell cycle, by promoting their phosphorylation and intracelluar localization and degradation of p27 [17,18]. Therefore, we wanted to determine the effect of silencing GRP-R on p21 expression in neuroblastoma. BE(2)-C cells were transfected with either control vector [BE(2)-C/shCON] or shRNA against GRP-R [BE(2)-C/shGRP-R] and synchronized by serum starvation for 24 h. Surprisingly, we found that p21 expression was significantly decreased in GRP-R silenced cells compared to cells transfected with control vector by Western blotting (Fig. 1A). Densitometric analysis showed that p21 expression in shGRP-R cells was reduced to 60% of shCON cells. Upon cell fractionation we found that p21 protein was predominantly localized in the nuclear compartment in both shCON and shGRP-R cells, and decreased expression was observed in shGRP-R cells (Fig. 1B).

3.2. GRP-R silencing increased p27 expression

Another key CDK inhibitor, p27, plays a major role in controlling cell cycle progression. Here, as opposed to p21 expression, p27 expression was upregulated in shGRP-R cells by Western blot analysis (Fig. 2A). Densitometry showed that p27 expression was 3-fold higher in shGRP-R cells than in shCON cells. Furthermore, we also found that p27 had a predominantly cytoplasmic localization in both shCON and shGRP-R cells, and also confirmed that the increased expression of p27 in both cytoplasmic and nuclear compartments in shGRP-R cells (Fig. 2B). These data demonstrated that p21 and p27 are differentially regulated by GRP-R signaling.

3.3. Silencing GRP oppositely modulated p21 and p27 expression

We previously reported that GRP activated the PI3K/AKT signaling pathway to promote cell cycle progression in neuroblastoma cells [5], whereas, silencing GRP increased the expression of p21 in SK-N-SH cells [19]. In order to confirm the differential regula-

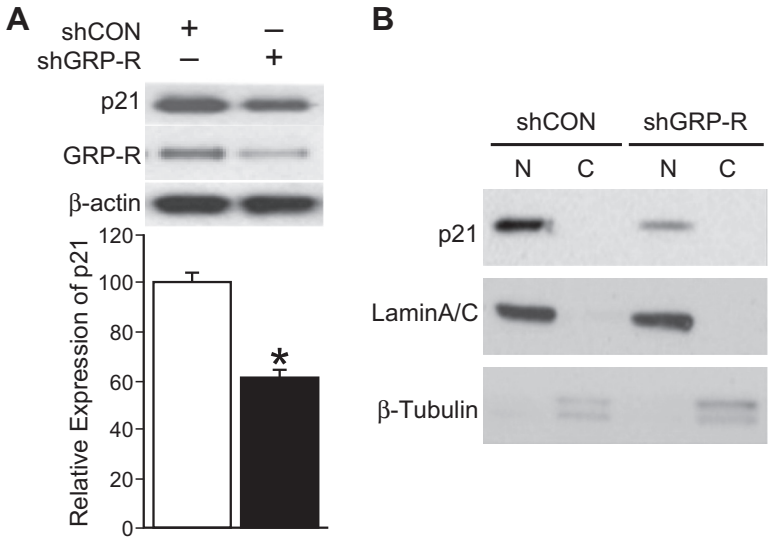

Fig. 1. GRP-R silencing decreased p21 expression. (A) GRP-R silenced BE(2)-C cells (shGRP-R) decreased p21 expression as measured by Western blot analysis. β-actin demonstrates equal protein loading. Densitometry analysis showed that p21 expression was reduced to 60% in shGRP-R cells (mean ± SEM; $^{*}p < 0.05$ vs. shCON) (B) shGRP-R decreased p21 nuclear levels, as demonstrated by cell fractionation analysis (N, nuclear; C, cytoplasmic). Lamin A/C and β-tubulin demonstrate the specificity of cell fractionation.

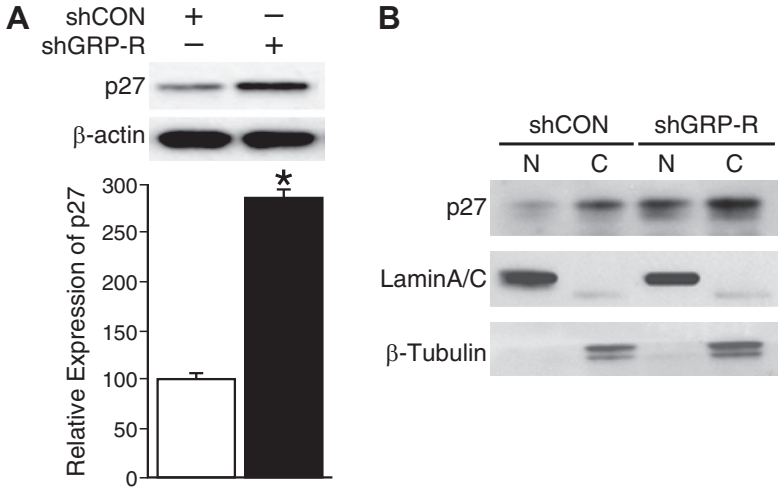

Fig. 2. GRP-R silencing increased p27 expression. (A) GRP-R silenced BE(2)-C cells (shGRP-R) increased p27 expression as measured by Western blot analysis. β-actin demonstrates equal protein loading. Densitometry analysis showed that p27 expression was increased by 3-fold in shGRP-R cells (mean ± SEM; $^{*}p < 0.05$ vs. shCON). (B) shGRP-R increased p27 nuclear levels, as demonstrated by cell fractionation analysis (N, nuclear; C, cytoplasmic). Lamin A/C and β-tubulin demonstrate the specificity of cell fractionation.

tions of GRP-R signaling pathway on p21 and p27, we next examined the expressions of p21 and p27 using tetracycline-inducible GRP silencing in BE(2)-C cells. We found that doxycycline-induced silencing of GRP also regulated p21 and p27 expression in a manner similar to GRP-R silencing. Treatment with doxycycline for 48 h downregulated the expression of p21, while the expression of p27 was upregulated (Fig. 3A, *left panel*). GRP silencing was confirmed with RT-PCR (Fig. 3A, *right panel*). Densitometric analysis demonstrated that the expression of p21was reduced by 75% after doxycycline treatment, whereas, p27 expression was increased by 4-fold after doxycycline treatment (Fig. 3A). Furthermore, immunofluorescent staining demonstrated that the fluorescent signal intensity of p27 was enhanced after doxycycline treatment (Fig. 3B). Taken together, our data suggest that silencing the ligand, GRP, had a similar effect to receptor (GRP-R) silencing with respect to regulation of p21 and p27 in neuroblastoma cells.

3.4. PTEN mediated GRP/GRP-R-induced p21 and p27 expression

We have previously shown GRP-R overexpression decreases PTEN levels [1]. Others have demonstrated that tumor suppressor PTEN and p27 cooperate in prostate cancer [20], potentially by directly interacting in the cytoplasm [21]. Because the loss of PTEN affects cell cycle progression by downregulation of p27 at the protein level, p27 was deemed as a downstream target of PTEN tumor suppressor [14]. In this study, we confirmed that PTEN was upregulated by ~180% in GRP-R silenced BE(2)-C cells (Fig. 4A). Furthermore, PTEN protein level was also increased by ~150% after doxycycline-induced GRP silencing in BE(2)-C cells (Fig. 4B). Immunofluorescent staining showed that increased PTEN protein expression was observed after doxycycline treatment in BE(2)-C/shGRP cells (Fig. 4C). We next performed cell fractionation and found that PTEN was mainly localized in cytoplasm along with

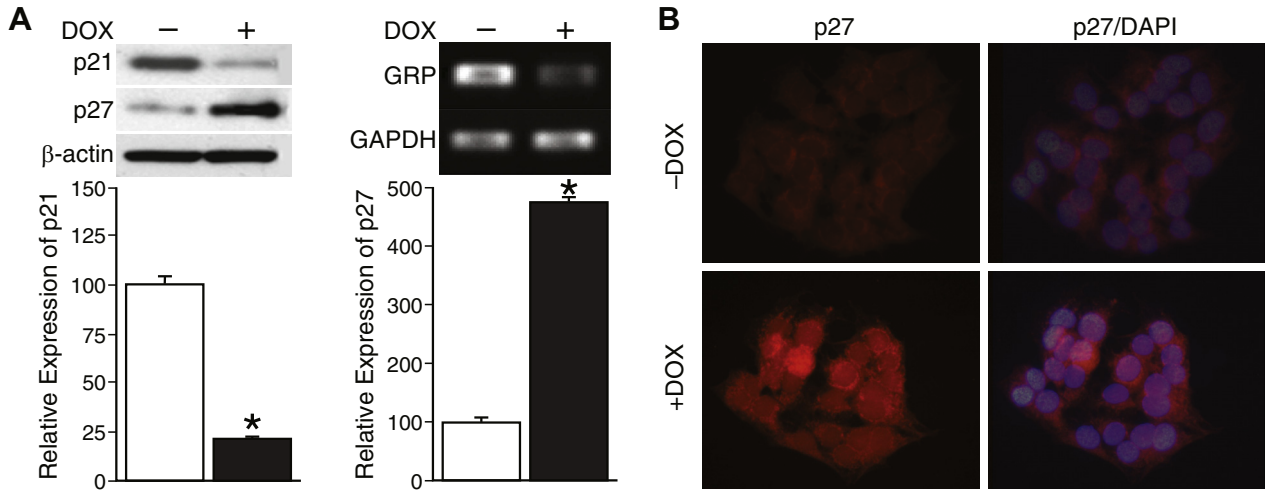

Fig. 3. Silencing GRP differentially induced p21 and p27 expression. (A) Doxycycline-induced GRP silencing resulted in decreased p21 protein levels, but increased p27, as measured by Western blotting in BE(2)-C cells. β-actin demonstrates equal protein loading. Densitometry analysis showed p21 expression was reduced to 25%, and p27 expression was increased by 4-fold after doxycycline. GRP silencing was confirmed by RT-PCR (mean ± SEM; $^{*}p < 0.05$ vs. no DOX). (B) Immunofluorescent staining confirmed the increases in p27 expression after GRP silencing (*bottom left panel*).

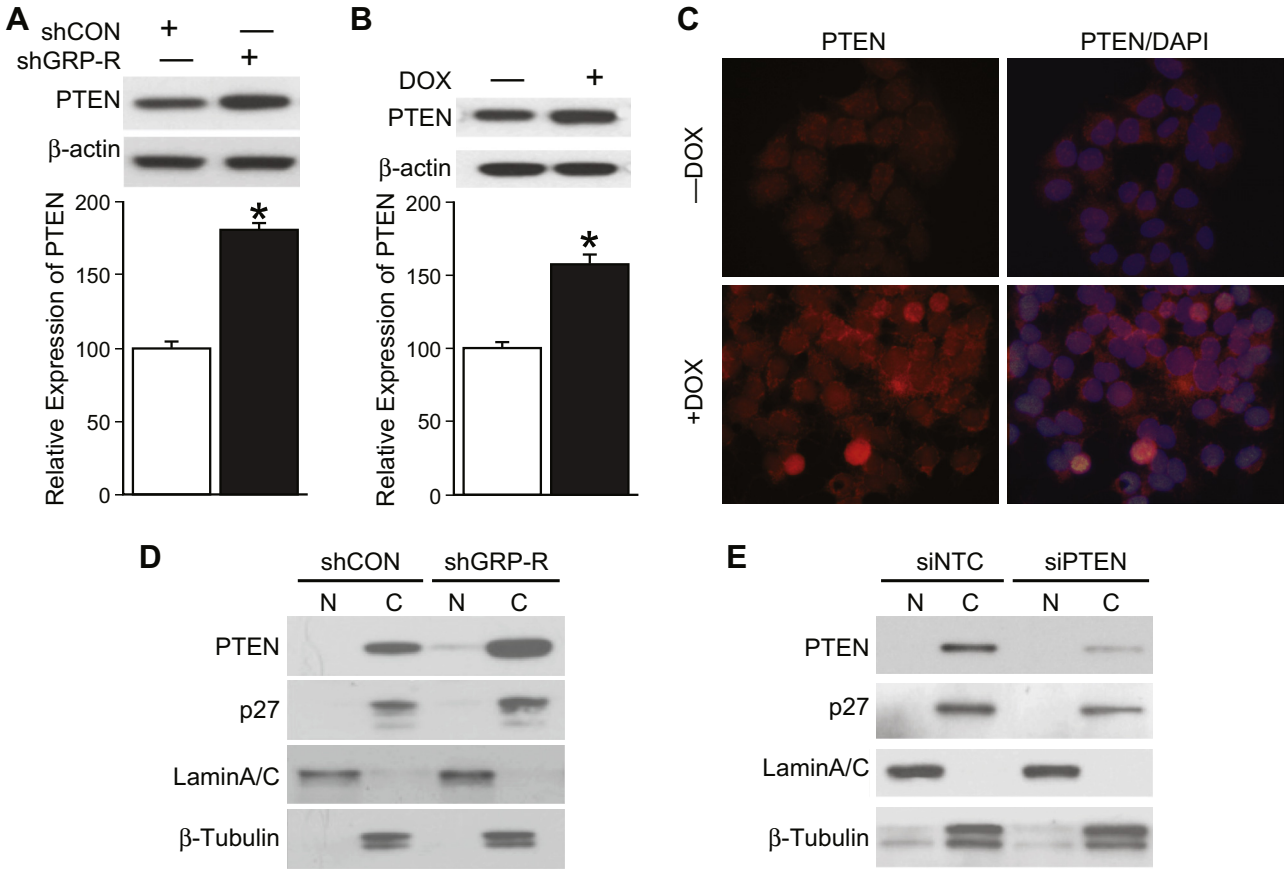

Fig. 4. GRP/GRP-R silencing induced PTEN and p27. (A) PTEN expression was increased in GRP-R silenced BE(2)-C cells (mean ± SEM; $^{*}p < 0.05$ vs. shCON). (B) PTEN expression was enhanced by doxycyline-induced GRP silenced BE(2)-C cells (mean ± SEM; $^{*}p < 0.05$ vs. no DOX). (C) Immunofluorescence staining showed increased expression of PTEN after doxycycline treatment (*bottom left panel*). (D) Subcellular distribution of PTEN was predominantly cytoplasmic, which was correlative to p27. Lamin A/C and β-tubulin demonstrate the specificity of cell fractionation. (E) GRP-R silenced BE(2)-C cells were transfected with non-targeting control siRNA (siNTC) and PTEN specific siRNA (siPTEN). p27 expression was decreased following PTEN silencing in cytoplasm of shGRP-R cells, as demonstrated by cell fractionation analysis (N, nuclear; C, cytoplasmic). Lamin A/C and β-tubulin demonstrate the specificity of cell fractionation.

p27 (Fig. 4D). Our results demonstrated that GRP/GRP-R signaling inhibition resulted in an increase in PTEN expression in addition to p27. In order to understand the correlation of PTEN and p27 in cells, we transiently silenced PTEN using siRNA in BE(2)-C/

shGRP-R cells. We found that PTEN silencing also decreased p27 expression. Moreover, we also found that PTEN co-localized with p27 in cytoplasm (Fig. 4E). Thus, our data indicate that PTEN mediates the regulation of GRP/GRP-R signaling on p27 in neuroblastoma.

4. Discussion

The molecular mechanisms of GRP/GRP-R-mediated neuroblastoma tumorigenesis have not been well characterized. We have previously demonstrated that PI3K/PTEN signaling axis plays critical roles in neuroblastoma [2]. In this present study, we sought to further understand PTEN-mediated GRP/GRP-R regulation on CDK inhibitors, p21 and p27. Using either stably transfected cells or inducible silencing systems, we found that GRP/GRP-R differentially regulated expressions of p21 and p27. p21 was decreased in both GRP and GRP-R silenced cells, while p27 expression was increased under the same conditions. PTEN expression was negatively induced by GRP and GRP-R, and also correlated with p27 expression.

PTEN, an endogenous negative regulator of PI3K, is genetically mutated or there is loss of PTEN expression in various advanced cancers. PTEN regulates PI3K signaling by dephosphorylating the lipid signaling intermediate PIP3 in cytoplasm and membrane, but PTEN may have additional phosphatase-independent activities, as well as other functions in the nucleus [4]. Cancer cells typically have low nuclear to cytoplasmic ratio of PTEN. In the nucleus, PTEN can mediate cell cycle arrest and growth inhibition via downregulation of MAPK and cyclin D1, while in the cytoplasm, PTEN can downregulate AKT activity and upregulate p27 resulting in caspase-mediated apoptosis [22]. We have previously reported that PTEN is highly expressed in ganglioneuroblastoma with differentiated cells in comparison to undifferentiated neuroblastomas [1]. GRP-R silencing inactivated the PI3K/AKT pathway and markedly increased PTEN expression [2]. In this study, we further demonstrated that silencing GRP, the specific ligand for GRP-R, resulted in a similar increase in PTEN expression, thus indicating that PTEN is a key mediator of GRP/GRP-R signaling in neuroblastoma tumorigenesis. Importantly, we also found that p27 is co-expressed with PTEN in the cytoplasm of neuroblastoma cells. Thus, inhibition of GRP/GRP-R signaling increased p27, which may cooperate with PTEN to suppress tumor growth by inducing cell death in a manner independent of cell cycle arrest.

Although both p21 and p27 are regulated by PI3K/AKT signaling, the results from our study suggest that p27 is the key regulator mediating inhibition on tumor growth after GRP/GRP-R silencing, and PTEN may also play a critical role in this regulation. p27 CDK inhibitor plays a major function in the control of cell cycle, cell migration and apoptosis, and is considered as a critical tumor suppressor. The absence, mislocalization, or dysfunction of p27 may lead to cancer development and/or may affect the outcome of anti-cancer therapies [23–26]. Although we did observe nuclear localization of p27 in this study, increased levels of cytoplasmic p27 by targeting GRP/GRP-R may be an important novel function as a tumor suppressor along with PTEN by yet unknown mechanisms.

In summary, we demonstrate that inhibition of GRP/GRP-R differentially regulates the expression of p21 and p27 in neuroblastoma cells. PTEN is a critical downstream effector of GRP/GRP-R signaling pathway and mediated the expression of p27. Our results show that the balance between PI3K and PTEN is important in neuroblastoma tumorigenesis. Furthermore, our study has discerned the molecular mechanisms downstream of GRP/GRP-R/PI3K and supports the importance of targeting GRP/GRP-R in providing a novel therapeutic strategy in neuroblastoma.

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