Epigallocatechin-3-gallate promotes all-trans retinoic acid-induced maturation of acute promyelocytic leukemia cells via PTEN

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Received May 21, 2017; Accepted July 25, 2017

DOI: 10.3892/ijo.2017.4086

Abstract. Acute promyelocytic leukemia (APL) is a distinctive subtype of acute myeloid leukemia (AML) in which the hybrid protein promyelocytic leukemia protein/retinoic acid receptor α (PML/RARα) acts as a transcriptional repressor impairing the expression of genes that are critical to myeloid cell mutation. We aimed at explaining the molecular mechanism of green tea polyphenol epigallocatechin-3-gallate (EGCG) enhancement of ATRA-induced APL cell line differentiation. Tumor suppressor phosphatase and tensin homolog (PTEN) was found downregulated in NB4 cells and rescued by proteases inhibitor MG132. A significant increase of PTEN levels was found in NB4, HL-60 and THP-1 cells upon ATRA combined with EGCG treatment, paralleled by increased myeloid differentiation marker CD11b. EGCG in synergy with ATRA promote degradation of PML/RARα and restores PML expression, and increase the level of nuclear PTEN. Pretreatment of PTEN inhibitor SF1670 enhances the PI3K signaling pathway and represses NB4 cell differentiation. Moreover, the induction of PTEN attenuated the Akt phosphorylation levels, pretreatment of PI3K inhibitor LY294002 in NB4 cells, significantly augmented the cell differentiation and increased the expression of PTEN. These results therefore indicate that EGCG targets PML/RARα oncoprotein for degradation and potentiates differentiation of promyelocytic leukemia cells in combination with ATRA via PTEN.

Introduction

Acute promyelocytic leukemia (APL) accounts for 10-15% of all cases of acute myeloid leukemia (AML) (1) and is characterized by a specific chromosomal translocation t(15;17) that fuses the promyelocytic leukemia gene (PML) to the retinoic acid receptor α gene (RARα), resulting in the translation of fusion proteins PML/RARα and RARα/PML (2,3). Pharmacological doses of all-trans retinoic acid (ATRA) produced clinical remission in APL patients by inducing the maturation of promyelocytes and the degradation of the PML/RARα protein (4,5). Nevertheless, ATRA does not eliminate the malignant myeloid clone in APL, and most relapsed APL patients are resistant to further treatment with ATRA (6). Therefore, we need to evaluate the combination of ATRA with other agents to work out a solution to drug resistance and harmful side-effects.

Epigallocatechin-3-gallate (EGCG), a principal antioxidant derived from green tea, has been shown to block each stage of carcinogenesis by modulating the signal transduction pathways involved in cell proliferation, transformation, differentiation, apoptosis, metastasis and invasion (7-10). Studies have shown that EGCG has anticancer effects in hematopoietic malignancy, and several mechanisms have been proposed for EGCG-induced cell death, including suppression of anti-apoptosis protein, VEGF receptor and inhibition of radical oxygen species (ROS) production (11-13). Recently, it was found that EGCG could suppress the expression of phosphorylated protein kinase (p-Akt) and phosphorylated serine/threonine-protein kinase mTOR (pmTOR) via phosphatase and tensin homolog (PTEN) to regulate the phosphatidylinositol 3-kinase (PI3K)/Akt/mTOR pathway, reducing proliferation and inducing apoptosis of cancer cells (14). Moreover, EGCG effectively induced apoptosis of APL cells through induction of the intrinsic apoptotic pathway and degradation of PML/RARα fusion protein (15,16).

PTEN is often lost or inactivated in multiple solid tumor types consisting of prostate, breast, thyroid, and endometrial tumors, and others, and is a critical regulator of the PI3K/Akt signaling pathway (17-19). Catalyzing the conversion of the membrane lipid second messenger phosphatidylinositol (3,4,5)-trisphosphate (PtdIns(3,4,5)P3) (PIP3) to PIP2, results in the inhibition of PI3K signaling in mutants lacking functional PTEN, suppressing hyperproliferation and releasing differentiation arrest (20-22). ATRA-mediated differentiation of the APL cell lines NB4

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Key words: PTEN, epigallocatechin-3-gallate, all-trans retinoic acid, PML/RARα, differentiation

and HL-60 showed that commercial PI3K and Akt inhibitors affect not only proliferation, but also the differentiative property of leukemia cells (23). ATRA-induced differentiation of HL-60 cells increased PTEN expression. Remarkably, ubiquitinylation of PTEN at specific lysine residues regulates its nuclear-cytoplasmic partitioning (24-26). Treatment with ATRA has been shown to trigger PML/RARα degradation and restores PML-NBs, where PML plays an essential role in the regulation of the tumor suppressive function of PTEN through ubiquitin carboxyl-terminal hydrolase 7 (USP7). Through restoration of nuclear PTEN, Akt has been shown to be antagonized, causing apoptosis and the production of differentiation stimuli (27,28).

The aforementioned findings prompted us to investigate whether EGCG could enhance ATRA induced APL cell line differentiation via PTEN. The results demonstrated that EGCG induced NB4 cell apoptosis by enhancing the expression of PTEN. Inhibiting PTEN levels resulted in a lower level of cell differentiation. Moreover, we found that a combination of ATRA with EGCG augmented cell differentiation in comparison with treatment with ATRA only.

Materials and methods

Cell lines and cell culture. The human AML cell lines, HL-60, NB4 and THP-1 were stored in our own laboratory, and cultured in RPMI-1640 medium (Gibco-Life Technologies, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS; Gibco, Melbourne, Australia) in an environment with 5% CO_2 at 37˚C.

Cell viability and proliferation. NB4 cells were seeded into 96-well plates with antibiotics-free RPMI-1640 media complemented with 10% FBS. For experimental purposes, cells were seeded at a density of $1x10^4$ cells/well and treated with 1 μM/ml ATRA [dissolved in 0.1% dimethyl sulfoxide (DMSO)] and EGCG (5, 10 and 15 μM, respectively) either alone or in combination for 72 h, and 10 μl Cell Counting kit-8 (CCK-8; 7Sea Cell Counting kit; Sevenseas Futai Biotechnology, Co., Ltd., Shanghai, China) was added to each well. After incubating for 2 h, the absorbance of each well was measured at 450 nm using a spectrophotometer (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Western blot analysis. Cells in each group were washed with ice-cold phosphate-buffered saline (PBS) three times, the supernatant was discarded and cells were lysed using ice-cold radioimmunoprecipitation assay (RIPA) lysis buffer containing protease inhibitor phenylmethanesulfonyl fluoride (PMSF), phosphatase inhibitor NaF and Na_3VO_4. The protein concentration was measured with the BCA protein assay kit. PTEN inhibitor SF1670 and PI3K inhibitor were purchased from Selleck Chemicals (Houston, TX, USA). Primary antibodies: PTEN (ab32199; 1:1,000; Abcam, Cambridge, UK), PML (EPR1768; 1:1,000; Abcam), RARα (1:1,000; Santa Cruz Biotechnology, Santa Cruz, CA, USA), Akt (ab32505; 1:1,000; Abcam), p-Akt (1:1,000; Cell Signaling Technology, Inc., Danvers, MA, USA), p21 (1:1,000; Wanleibio, Co., Ltd., Beijing, China), β-actin (1:1,000; Beijing Zhongshan Golden Bridge Biotechnology, Co., Ltd., Beijing, China).

Cell morphological staining. After 72 h of treatment, cells were collected and washed with pre-cooled PBS three times and resuspended in fresh PBS. Cell suspension (10 μl) was daubed onto glass slides, and then the air dried slides were stained with Wright-Giemsa staining fluid. For nitro blue tetrazolium (NBT) staining, cells were collected after 72 h and resuspended in fresh RPMI-1640 medium supplemented with 10% FBS, and $3x10^5$ cells/well were seeded on 96-well plates and combined with 200 μl mixture with 0.2% NBT and 240 μg/ml 12-*O*-tetradecanoylphorbol-13-acetate (TPA), followed by incubation for 1 h (37˚C, 5% CO_2). Samples were centrifuged at 1000 rpm for 5 min and 200 μl DMSO was added to each well, followed by shaking for 20 min. Finally, 10 μl of CCK-8 was added to each well and the absorbance was measured at 570 nm (29,30).

Respiratory burst assay. As a measure of differentiation, the respiratory burst assay for detecting hydrogen peroxidase was used. Cells were collected after 72 h, resuspended in fresh RPMI-1640 medium supplemented with 10% FBS and seeded on 96-well plates. PMA was added at a final concentration of 200 ng/ml to the cells ($3x10^5$ cells/well). Immediately, 10 μl of CCK-8 was added to each well, with each experimental group paired with three parallel control groups, and the cells were incubated for 1 h (37˚C, 5% CO_2) prior to measuring the absorbance at 412 nm (31).

Analyses of cell differentiation marker by flow cytometry. For detection of the cell differentiation antigen, CD11 antigen-like family member B was used (CD11b), after 72 h of treatment, the cells were collected ($1x10^6$/group) and washed with three times with pre-cooled PBS, then incubated with phycoerythrin (PE) conjugated CD11b antibody (12011342; eBioscience, Inc., San Diego, CA, USA) at 4˚C for 30 min in the dark (32). The cells were then analyzed using flow cytometry (BD FACSVantage; BD Biosciences, San Jose, CA, USA) and CellQuest Pro software version 5.1 (BD Biosciences).

Indirect immunofluorescence assay. Cells were fixed with 4% paraformaldehyde for 20 min, subsequently, permeabilized with 0.1% Triton X-100 (in PBS) for 15 min, and then blocked in 10% goat serum (in PBS) for 30 min at room temperature. Slides were then incubated overnight with the indicated primary antibodies. Secondary goat antibody against rabbit-IgG-TRITC (1:200; Beijing Zhongshan Golden Bridge Biotechnology) was used to detect rabbit IgG for 1 h at room temperature. The nuclei were stained using DAPI at room temperature. Finally, coverslips were immobilized by 70% glycerol and viewed under a fluorescence microscope (Nikon, Tokyo, Japan).

Statistical analysis. All data were performed using the SPSS 17.0 software (SPSS, Inc., Chicago, IL, USA). Results are represented as the mean ± SD. The Student's t-test was used for statistical analysis.

Results

EGCG in combination with ATRA enhances NB4 cell differentiation. Treatment of NB4 cells with increasing concentrations

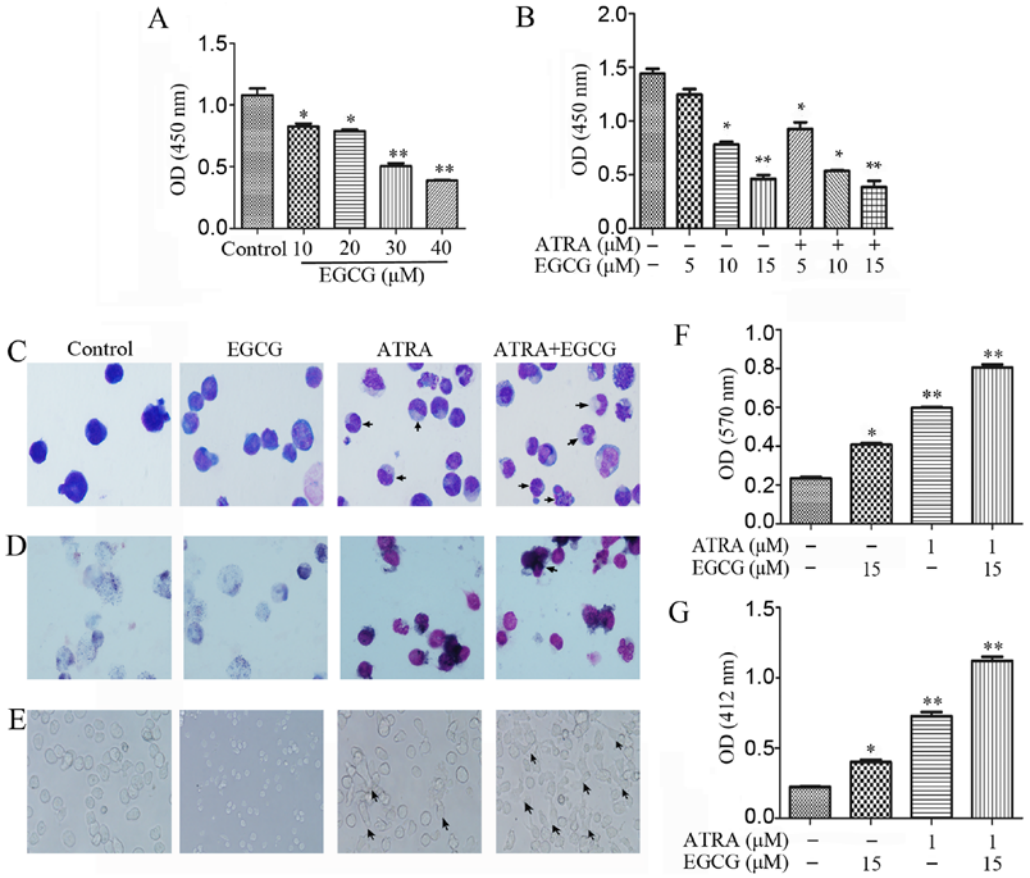

Figure 1. EGCG in combination with ATRA enhances NB4 cell differentiation. (A) Growth inhibition of NB4 cells when treated with increasing concentrations of EGCG (0-40 μM) for 48 h (n=3) using the CCK-8 assay; (B) NB4 cells following single or combined exposure to ATRA (1 μM) and EGCG for 72 h (n=3); (C) morphology of Wright-Giemsa stained NB4 cells treated with EGCG and/or ATRA. Magnification, x40. (D) NBT staining of NB4 cells showing an increased proportion of differentiated cells following 3 days of treatment with ATRA alone or in combination with EGCG. Magnification, x40. (E) After treatment for 3 days, NB4 cells attached to the culture vessels (arrowheads). Magnification, x40. (F and G) Functional assessment of NB4 cell differentiation as evaluated using the NBT reduction assay and respiratory burst assay on day 3 of treatment. Mean values with different symbols indicates significant differences from the control group. Data were analyzed using a Student's t-test; *P<0.05 and **P<0.01.

(0-40 μM) of EGCG for 48 h resulted in diminished proliferation (Fig. 1A). To investigate the effects of EGCG in the presence of ATRA, we measured cell viability following treatment with different concentrations of EGCG combined with 1 μM ATRA (Fig. 1B). It has been shown previously that ATRA induced differentiation of APL cells instead of promoting proliferation. To verify the phenomenon, we investigated the differentiation of NB4 cells in several ways. Wright-Giemsa staining was used for morphological analysis, with the results indicative of augmented differentiation both with ATRA alone and when combined with EGCG (Fig. 1C). The NBT reduction assay produced high staining intensities for these treatments, suggestive of an advanced maturation status (Fig. 1D and F). Moreover, we observed the adherent status in a subpopulation of cells following both treatment with ATRA alone or ATRA and EGCG (Fig. 1E). Respiratory burst activity was measured to evaluate the oxidation respiratory function of the differentiated cells. We observed that respiratory burst activity was higher in the combined treatment than with ATRA alone, suggesting an advanced cell maturation (Fig. 1G).

Enhancement of ATRA-induced upregulation of PTEN and its redistribution by EGCG applied to differentiation in NB4 cells. After 72 h of treatment with EGCG and ATRA, NB4 cells were examined by flow cytometric analysis of the myeloid differentiation marker CD11b. The combined treatment significantly increased CD11b level in comparison with ATRA alone (Fig. 2A and B). For the treatment of NB4 cells with 1 μM ATRA for 1, 2 and 3 days, the protein expression levels of PTEN, CD11b and CCAAT-enhancer-binding protein beta (C/EBPβ) were increased in a time-dependent manner while the level of Akt phosphorylation was decreased (Fig. 2C). In addition, we consistently observed that the increased PTEN expression level was closely related to CD11b expression with both ATRA alone and the combined treatment, and the same result was produced in HL-60 and THP-1 cells (Fig. 2E). This suggests that PML and PML nuclear body (PML-NB) regulation of PTEN localization may have relevance in APL. PML/RARα inhibits PTEN expression in NB4 cells. Inhibition of proteasome function using proteases inhibitor MG132 rescued PTEN from PML/RARα degradation (Fig. 2D). It is known that polyubiquitination of PTEN leads to its degradation in the cytoplasm, while monoubiquitination is essential for important cell functions, including cell growth, tumor suppression, cell differentiation and migration (24,26). Compared to ATRA alone, the combined treatment resulted in increased PML expression and de-ubiquitinylation of PTEN was inhibited, augmenting

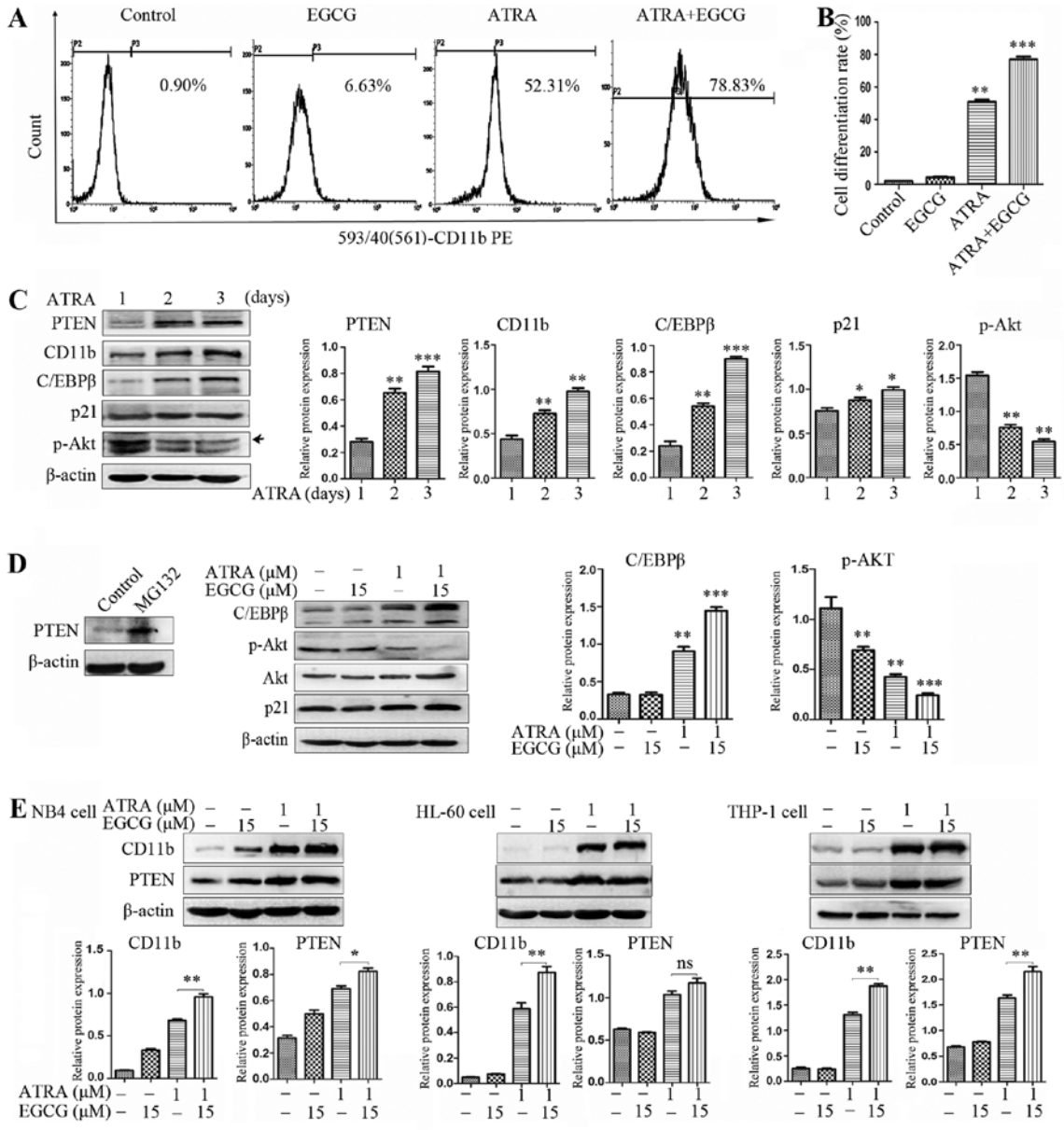

Figure 2. Enhancement of ATRA-induced upregulation and redistribution of PTEN by EGCG applied to cell differentiation in NB4 cells. (A and B) NB4 cells were treated with 1 μM ATRA or 15 μM EGCG or both, and the percent of differentiated cells was determined by measuring CD11b expression; (C) NB4 cells were treated with 1 μM ATRA for 24, 48 and 72 h, and the level of PTEN, CD11b, C/EBPβ and p-Akt were determined by western blot analysis. (D) Proteasome inhibition rescued PTEN expression (left panel). EGCG enhanced ATRA-induced C/EBPβ and p21 and blocked p-Akt (right panel); (E) NB4, HL-60 and THP-1 cells were treated with 1 μM ATRA or 15 μM EGCG for 3 days, the increased level of PTEN expression was closely related to CD11b expression in the combined treatment; *P<0.05, **P<0.01 and ***P<0.001 in comparison with the untreated cells.

the level of nuclear PTEN (Fig. 3A). Consistent with previous results, nuclear extracts had higher concentrations of PTEN than cytoplasmic extracts (Fig. 3B).

EGCG abrogates PML/RARα expression in NB4 cells. EGCG was shown to trigger PML/RARα degradation and restore PML function (Fig. 4A). The expression of PML/RARα and PML at the protein level was assessed in NB4 cells, where PML/RARα expression was decreased in cells receiving ATRA alone and the combined treatment, while the protein expression level of PML increased. However, EGCG treatment alone greatly abrogated PML/RARα protein expression in whole cell extracts (Fig. 4B).

PTEN catalyzes the conversion of PIP3 to PIP2, antagonizing PI3K signaling, inducing cell differentiation and anti-proliferation. PI3K signaling regulates diverse cellular process, including cell proliferation and survival, reducing the activity of PTEN (33). Neutrophil functions, such as phagocytosis, oxidative bursting, polarization, and chemotaxis were augmented after treatment with PTEN inhibitor SF1670 (34). In the present study, we used PTEN inhibitor SF1670 to enhance the PI3K signaling pathway and repress cell differentiation (Fig. 5A). To further assess the potency of PTEN inhibition of the PI3K/Akt pathway, PI3K inhibitor LY294002 was used to pretreat NB4 cells, significantly augmenting the cell differentiation and reducing the expression of p-Akt (Fig. 5B).

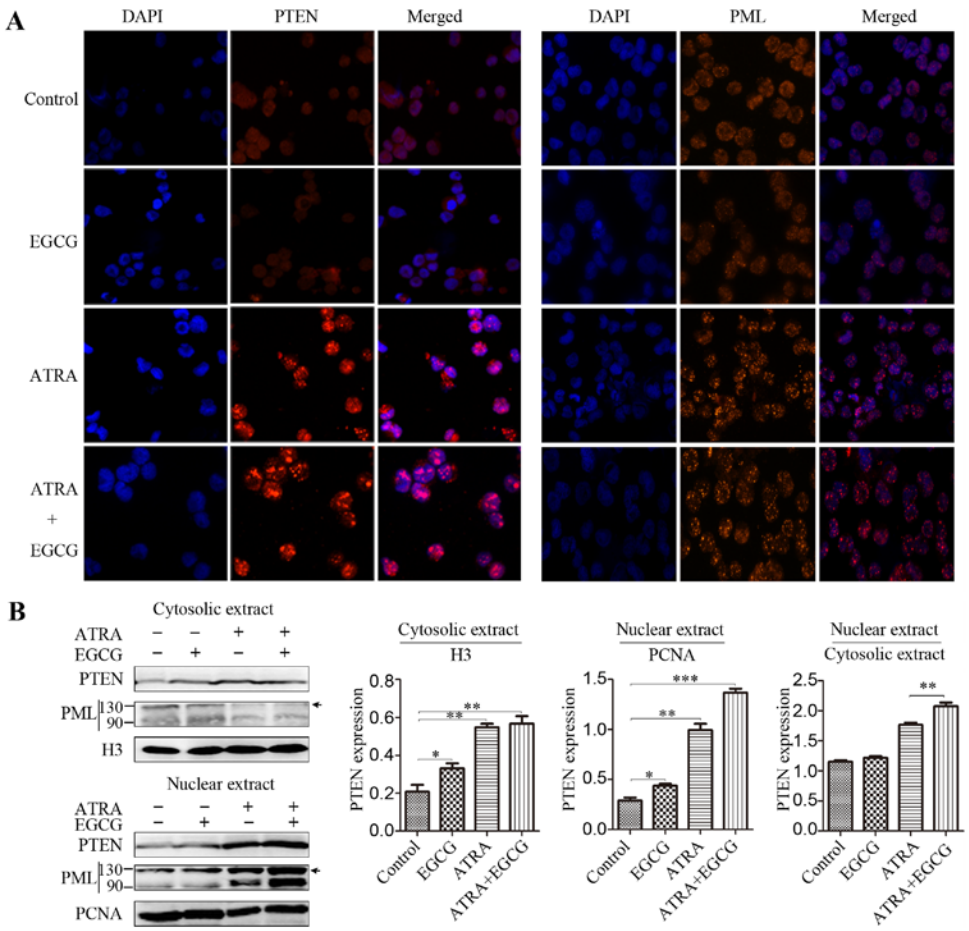

Figure 3. Enhancement of ATRA-induced upregulation and redistribution of PTEN by EGCG applied to cell differentiation in NB4 cells. (A) In NB4 cells, combination drug treatment increased the expression of PML (right panel) and inhibited deubiquitination of PTEN, augmenting the level of nuclear PTEN compared to treatment with ATRA alone (left panel). Magnification, x40. (B) Immunoblot analysis was applied to both the cytosolic and nuclear fractions following treatment with ATRA and/or EGCG for 72 h in NB4 cells. The combination treatment resulted in higher protein expression levels of PTEN, PML and RARα in nuclear extracts than in the cytoplasm. H3 served as a loading control for the cytosolic fraction; PCNA served as a loading control for the nuclear fraction. *P<0.05, **P<0.01 and ***P<0.001 in comparison with the untreated cells.

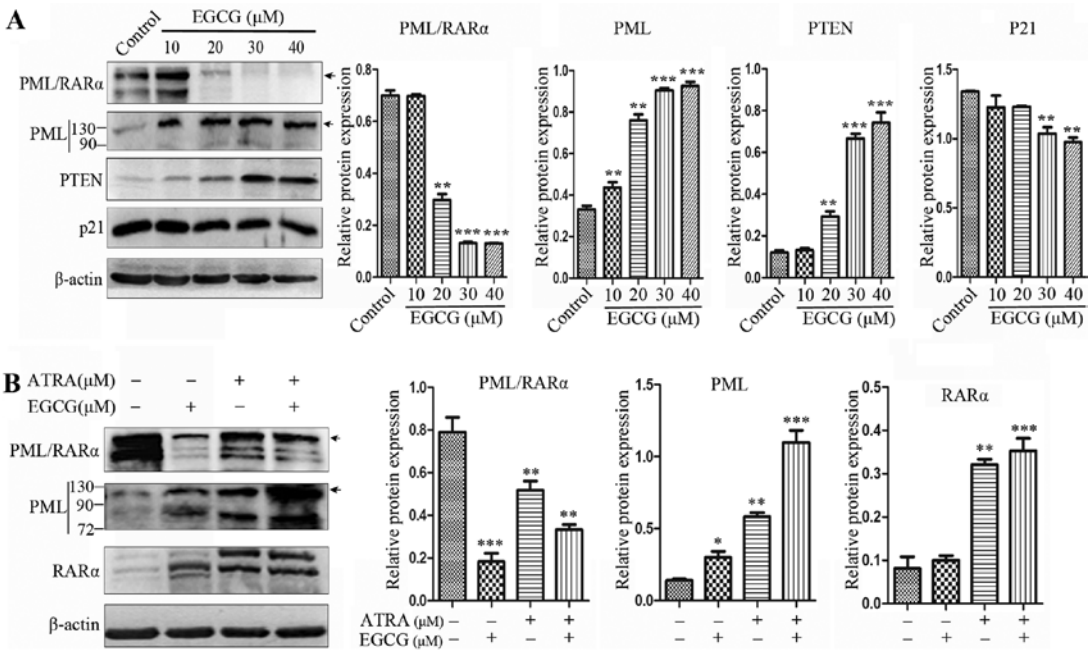

Figure 4. EGCG abrogates PML/RARα expression in NB4 cells. (A) The PML/RARα oncoprotein was degraded in NB4 cells treated with increasing concentrations of EGCG for 48 h. The expression of p21 was increased at the protein level in a dose-dependent manner; (B) immunoblot analysis of PML/RARα, PML and RARα in whole cell extracts. *P<0.05, **P<0.01 and ***P<0.001 in comparison with the untreated cells.

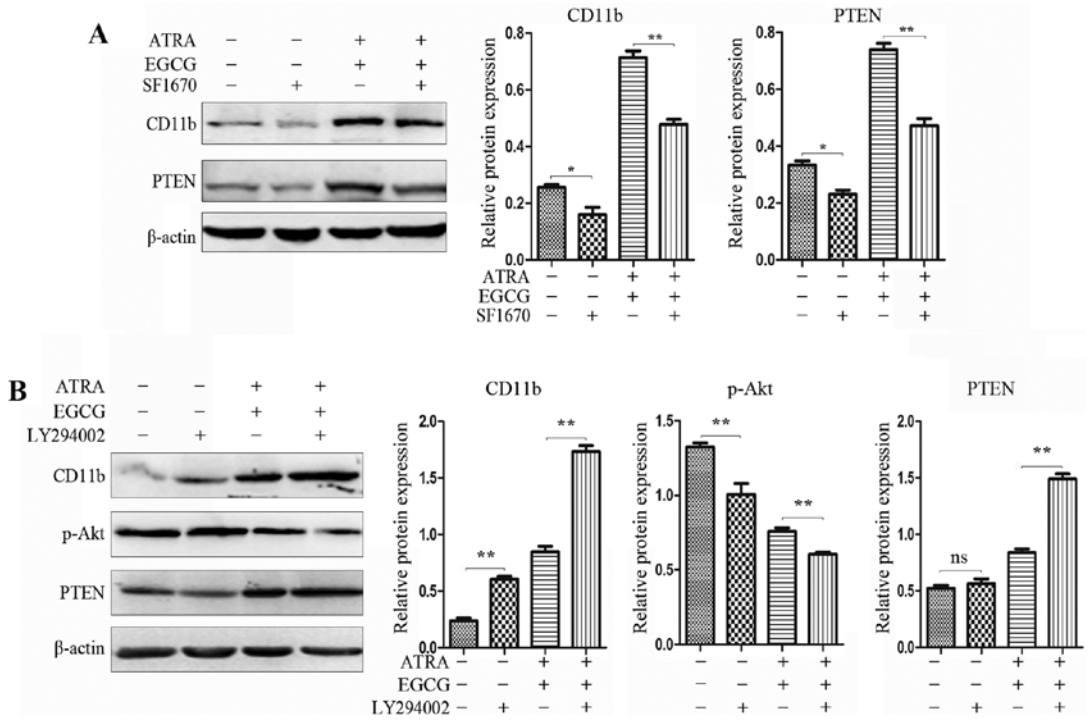

Figure 5. PTEN catalyzes the conversion of PIP3 to PIP2 antagonizing the PI3K signaling, inducing cell differentiation. (A) A total of 1.5 μM PTEN inhibitor SF1670 inhibited the expression of PTEN and decreased the level of CD11b; (B) 20 μM PI3K inhibitor LY294002, inhibited the expression of p-Akt. Moreover, the level of PTEN and CD11b were increased. *P<0.05 and **P<0.01 in comparison with the no inhibitors group.

Cytoplasmic PTEN is monoubiquitinylated by E3 ubiquitin-protein ligase (NEDD4) and subsequently translocated into the nucleus. Moreover, PTEN is further ubiquitinated in the cytoplasm and degraded, by the proteasome. After combination treatment with ATRA and EGCG, PML/RARα oncoprotein was degraded, restoring PML levels and, inhibiting HAUSP-mediated deubiquitinylation and nuclear export of PTEN. Nuclear PTEN can shuttle back to the cytoplasm, or after deubiquitination, remains nuclear and protected from cytoplasmic degradation. Importantly, nuclear PTEN is still able to antagonize the Akt signaling pathway and induces cell differentiation.

Discussion

APL was successfully treated with ATRA that triggers PML/RARα fusion protein degradation and induces the maturation of promyelocytes. However, a large proportion of patients with APL still face relapse. Therefore, novel agents are essential to improve the outcomes for APL patients. Previous studies showed that EGCG induces hematopoietic malignant cell apoptosis by the production of ROS *in vitro*. Moreover, EGCG is an ATP-competitive inhibitor of both PI3K and mTOR, restraining cell proliferation and Akt phosphorylation at Ser473 in human breast adenocarcinoma (MDA-MB-231) and lung carcinoma (A549) cell lines (35). Collectively, the body of research strongly suggests that EGCG may represent a potential target for treatment of pancreatic cancer via PTEN activation regulating the PI3K/Akt/mTOR pathway (36). Research has shown that EGCG in synergy with ATRA upregulated the expression of some differentiation markers and differentiation-inducing genes, the enhancing effects of co-treatment recommended additional mechanism (15).

PTEN is one of the molecular pathways involved in the balance between proliferation, differentiation and apoptosis during hematopoiesis. It inhibits proliferation and promotes differentiation as a tumor suppressor, including acute promyelocytic leukemia (37). The present study established an essential role for PTEN in the balance between proliferation and differentiation of blood cells. However, little is known about the molecular mechanism of cell differentiation regulating by PTEN. In this study, we found that EGCG potentiated NB4 cell differentiation in combination with ATRA, at least in part, via the actions of PTEN. We showed that nuclear PTEN is capable of inducing cell differentiation in leukemia blasts in response to combination treatment. Thus, it is tempting to assume a dual function for PTEN as a mediator of cell differentiation in maturing APL cells. Cytoplasmic PTEN mainly down-modulates Akt activation via regulation of PIP3 levels. In many leukemia cell lines, the PTEN expression was suppressed, which would contribute to activating PI3K/Akt signaling by suppressing the conversion of PIP3 to PIP2, resulting in hyper-proliferation and differentiation arrest. However, nuclear PTEN is protected from degradation, which plays a direct role in the chromosome stability, DNA repair and cell cycle arrest. Both residues facilitate the PTEN binding to the membrane, thereby suppressing anchorage-independent cell proliferation and tumor growth.

The present study highlights a role for PML and PML-NBs in the regulation of PTEN localization, where disruption of PTEN localization may have relevance in malignancies where PML and PML-NBs are compromised, as found in APL.

Treatment with ATRA or arsenic trioxide (ATO) triggers PML/RARα degradation and restores NBs, acting as part of a PML network to regulate PTEN deubiquitination. Both mono- and poly-ubiquitinated PTEN exist *in vitro* and *in vivo*, where mono-ubiquitination is essential for increasing protein stability and nuclear localization of mutant of PTEN (38). NEDD4 has both oncogenic (PTEN degradation) and tumor suppressive (PTEN shuttling) potential (26,39). Consistent with this study, we also provide evidence for abrogation of PML/RARα expression by EGCG alone in a different concentration set-up. We first investigated that the combination treatment can promote degradation of PML/RARα and restore PML expression. Partial repression of PML/RARα was observed in the combined treatment with ATRA, but the expression of PML was increased, and no differentiation blockade was observed in the combined treatment. Since PML can suppress the function of HAUSP, inhibiting the deubiquitination of PTEN and increasing the level of nuclear PTEN, we aim to accentuate that PML/ubiquitinated-PTEN/Akt signaling pathway is essential for NB4 cell differentiation.

In the present study, we assessed the combined activity of EGCG and ATRA on NB4 cell differentiation. It was determined that the two drugs in combination have strong synergistic effects whereby differentiation is stimulated. We found that PTEN protein was more strongly expressed in the nucleus than in the cytoplasm during NB4 cell differentiation and preformed the effects of PTEN and AKT on differentiation in acute promyelocytic leukemia NB4 cells. To this end, findings have suggested that PTEN inhibitor SF1670 and PI3K inhibitor LY294002 inhibited the basal level and combination treatment level of PTEN and PI3K, respectively, where the proportion of differentiation NB4 cells was changed.

Taken together, EGCG may represent a novel effective and safe drug for APL treatment, and could be used synergistically with ATRA to promote degradation of PML/RARα and restore PML expression, inhibiting the de-ubiquitination of PTEN and increasing the level of nuclear PTEN. Therefore, we believe that the PML/ubiquitinated-PTEN/Akt signaling pathway is essential for NB4 cell differentiation. Overall, our results report PTEN as a key player in both the cell death response and enhancement of neutrophil differentiation. Our next investigation is aimed at PTEN and PML to investigate the differentiation of APL cells.

Acknowledgements

The present study was supported by a grant from the National Natural Science Foundation of China (grant no. 81171658) and the Natural Science Foundation Project of CQ CSTC (grant no. 2011BA5037).

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