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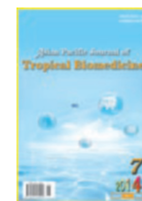

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Down-regulated expression of NPM1 in IMS-M2 cell line by (-)-epigallocatechin-3-gallate

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PEER REVIEW

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Comments

This is a good and useful study in which the authors have evaluated the down-regulated expression of NPM1 in IMS-M2 cell line by EGCG. The results obtained in this work clearly suggested that EGCG was good for treatment of AML patients.
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ABSTRACT

Objective: To investigate the inhibited effect of epigallocatechin-3-gallate (EGCG) on the expression of NPM1 in IMS-M2 cells harboring the *NPM1* mutations.
Methods: Cell proliferation assay was performed to test the effects of EGCG on cell growth of IMS-M2 cells harboring the *NPM1* mutations. Western blot analysis were performed to test the protein expression of NPM1, AKT, those associated with apoptosis.
Results: EGCG can down-regulate the expression of NPM1 in IMS-M2 cells harboring the NPM1 mutations. Moreover, EGCG also suppressed the cell proliferation and induced apoptosis in IMS-M2 cells.
Conclusions: The results suggested that EGCG could be considered as a reagent for treatment of AML patients with *NPM1* mutations.

KEYWORDS
NPM1, EGCG, IMS-M2, Apoptosis

1. Introduction

Nucleophosmin 1 (NPM1), one of the isoforms of NPM protein (also known as B23, numatrin or NO38), is a protein that shuttles between the nucleus and cytoplasm found at high levels in the granular regions of the nucleolus[1,2]. Many findings have revealed a complex scenario of NPM1 functions and interactions. Some main functions have been ascribed to NPM1: 1) promoting the biogenesis of the ribosome by acting as a chaperone that carries pre-ribosomal particles from the nucleolus to the cytoplasm and by facilitating the processing and maturation of ribosome RNA[1,3]; 2) controlling the duplication of the centrosome during the cell cycle[4]; 3) modulating the function of tumor-suppressor transcription factors, such as interferon regulatory factor 1 (IRF-1) and p53[5]; and regulating the function and stability

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of the p19^{ARF} tumor suppressor[6] and the apoptosis[7].

NPM1 is an essential protein, since the inactivation of the gene encoding for NPM1 in the mouse germ line leads to developmental defects that cause embryonic death in mid-gestation[8]. In humans, accumulating evidences suggest that NPM1 is directly implicated in the pathogenesis of cancer. NPM1 is over-expressed in solid tumors of diverse histological origin or is involved in tumor progression[9,10]. In several hematologic malignancies, the NPM1 locus is lost or translocated leading to the formation of oncogenic fusion proteins[11]. Moreover, *NPM1* is mutated in about one-third of adult patients with acute myeloid leukemia (AML), which makes *NPM1* mutations the most frequent genetic lesions so far that identified in *de novo* AML[12].

Mutations of *NPM1* in AML disrupt the nucleolar-localization signal, causing accumulation of NPM1 in the cytoplasm. AML with mutated NPM1 is generally characterized by good response to induction chemotherapy[13] and favorable prognosis (in the absence of a concomitant FLT3-ITD mutation)[14,15]. However, a significant number of cases with *NPM1*-mutated AML still show poor outcome, especially those associated with *FLT3-ITD* mutation and elderly patients. Currently, some possible approaches on the development of a targeted therapy for *NPM1*-mutated AML were attributed, which including: 1) interfering with the aberrant transport of the NPM1 leukemic mutant; 2) inhibiting the capability of the residual wild-type nucleophosmin and other nucleolar components to act as hub proteins for assemblement of the nucleolus; and 3) intervening on minimal residual disease (NPM1 mutant copy transcripts) before overt hematological relapse occurs (so-called pre-emptive therapy). Evaluating the activity of epigenetic drugs (*e.g.* 5-azacytidine) or agents acting on differentiation and apoptosis in *NPM1*-mutated AML is also warranted[14].

In this paper, we have demonstrated that epigallocatechin-3-gallate (EGCG) can down-regulate the expression of NPM1 in IMS-M2 cells harboring the *NPM1* mutations. Moreover, EGCG also suppressed the cell proliferation and induced apoptosis in IMS-M2 cells. We suggested that EGCG could be considered as a reagent for treatment of AML patients with *NPM1* mutations.

2. Materials and methods

2.1. Cell lines and culture conditions

IMS-M2 cells have been described previously[16]. Briefly, IMS-M2 was established from the bone marrow cells taken from a 59-year-old patient with AML (FAB M2), chromosome abnormalities of 48, XX, add (6) (q27), +8, inv(12) (p13q15), add (15) (q25), +add (15) (q25). This cell line harbors the mutation of *NPM1* gene and the fusion of *ETV6* to neurotrophin-3 receptor *TRKC*[16]. A leukemic cell lines MOLM13[17] with NPM1 wild-type[18] was used as control.

The cells were grown in RPMI 1640 medium (Sigma-Aldrich, Japan K.K., Tokyo, Japan) supplemented with 10% heat-inactivated fetal bovine serum (JRH Biosciences, Lenexa, KS, USA), 100 IU/mL penicillin, and 0.1 mg/mL streptomycin (Nakalai Tesque, Kyoto, Japan) in a humidified incubator of 5% CO_2 at 37 °C.

2.2. Reagents

A purified powder of EGCG was generously gifted by Dr. Yukihiko Hara (Japan). EGCG was dissolved in dimethylsulfoxide (DMSO) (Wako Pure. Chemical Industries, Osaka, Japan). Controlled cells were cultured with the same concentration of carrier DMSO as used in the highest dose of reagents. The concentration of DMSO was kept under 0.1% throughout all the experiments to avoid its cytotoxicity.

2.3. Cell proliferation assays

Cell proliferation was determined by trypan blue dye exclusion test as described previously[17]. Briefly, cells were seeded in 6-well plates at a density of 1×10^5 cells/mL in the presence of different concentrations of EGCG for 48 h. After the treatment, 10 μL of the cell suspension was mixed with 10 μL of 0.4% trypan blue, and alive cells were counted manually using a hemacytometer. Results were calculated as the percentage of the values measured when cells were grown in the absence of EGCG.

2.4. Morphologic assessment to detect apoptotic cells

For detecting fragmented nuclei and condensed chromatin, cells at a density of 1×10^5 cells/mL were treated with reagents. After indicated durations, cells were harvested and fixed onto slides by using a cytospin (Shandon, Shandon Southern Products Ltd., Cheshire, UK). Cells then were stained with Wright-Giemsa solution. Morphology of cells was observed under an inverted microscope.

2.5. Western blot analysis

Cells were plated onto 10 cm dishes at a density of 1×10^5 cells/mL in the presence of various concentrations of EGCG. After incubation for indicated durations, cells were collected and washed twice with PBS (-). Cells were then dissolved in a protein lysis buffer containing 5 mmol/L EDTA, 50 mmol/L NaF, 10 mmol/L $Na_2H_2P_2O_7$, 0.01% Triton X-100, 5 mmol/L HEPES, 150 mmol/L NaCl, 1 mmol/L Na_3VO_4, 1 mmol/L phenylmethylsulfonyl fluoride, and 75 μg/mL aprotinin on ice for 30 min with brief vortex of 4 times every 10 min. After centrifugation at 13 000 r/min at 4 °C for 10 min, total cell lysates were collected for western blot analysis. Protein samples were electrophoresed through a polyacrylamide gel and transferred to a Hypond-P membrane (Amersham, Buckinghamshire, UK) by electro-blotting. After washing,

the membrane was probed with antibodies and antibody-binding was detected using enhanced chemiluminescence ECL (Amersham). The following antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA): total Akt (sc-1618), anti-rabbit IgG-HRP (sc-2317) and anti-mouse IgG-HRP (sc-2031). Anti-actin (A2066) was from Sigma-Aldrich. Phospho-Akt (Ser473), caspase-3 and NPM antibody were from Cell Signaling Technology Japan (Tokyo, Japan). Anti-PARP antibody was from WAKO Chemicals (Osaka, Japan).

2.6. Statistical analysis

All datas were expressed as the mean±SD. Statistical analyses were done using Student's t-test, in which $P<0.05$ was the minimum requirement for a statistically significant difference.

3. Results

3.1. Growth-inhibitory effect of EGCG on IMS-M2 cells

First, to test on growth-inhibitory effect of EGCG, IMS-M2 cells were incubated either with the carrier DMSO alone (0 μmol/L EGCG) or with 5, 10, 20, 40 or 80 μmol/L EGCG for 48 h. Cell proliferation was evaluated using the trypan blue exclusion test. The effect of EGCG on cell growth has been demonstrated in various cancers. In IMS-M2 cells, the percent of cell proliferation showed that EGCG suppressed the cell growth of IMS-M2 (Figure 1). The effect of EGCG on IMS-M2 cells is dependent on cell density as coincide with the results observed in GIST cell lines[19]. At a density of 1×10^5 cells/mL and 1×10^4 cells/mL, IC_{50} of EGCG was around 20 and 5 μmol/L, respectively (Figure 1). The cell growth inhibition by EGCG diminished dramatically with increasing cell densities, similar to phenomena reported in colorectal carcinoma cells and GIST cell[19].

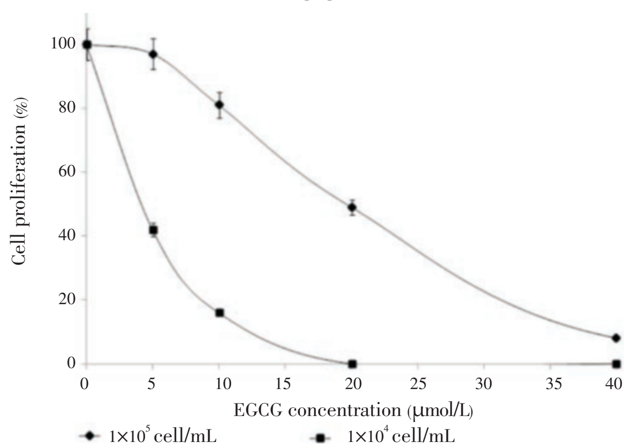

Figure 1. Effect of EGCG on cell proliferation of IMS-M2 cells.
IMS-M2 cells at a density of 1×10^5 cells/mL or 1×10^4 cells/mL were treated with 5, 10, 20 or 40 μmol/L EGCG or DMSO alone (0 μmol/L EGCG) as control for 48 h. The number of alive cells was counted after trypan blue exclusion test. Results were calculated as the percentage of the control values.

3.2. Down-regulation of NPM1 in EGCG-treated IMS-M2 cells

Next, we checked whether EGCG can affect on expression status of NPM1 protein. IMS-M2 cells were treated with different concentration of EGCG. After 8 h, the cells were harvested and extracted and then total cell lysates were subjected to western blot analysis. Interestingly, the expression of NPM1 was suppressed in EGCG-treated IMS-M2 cells in dose-dependent manner (Figure 2A). The time course was performed with the EGCG concentration and fixed at 60 μmol/L for 3 and 8 h. The results shown that EGCG also inhibited the NPM1 expression in a time dependent manner (Figure 2B). Moreover, exposing MOLM13 cells that harboring wild-type NPM1 to different concentration of EGCG revealed that EGCG could not inhibited the expression of NPM1 (Figure 2C).

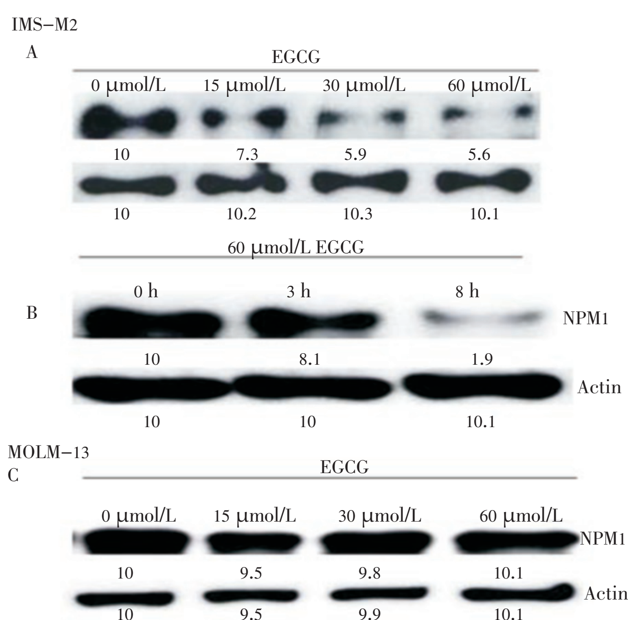

Figure 2. Down-regulation of NPM1 expression in FLT3-mutated cells.
Panel A and B showed the results of down-regulation of NPM1 expression by EGCG treatment in IMS-M2 cells as well as MOLM13 control cells shown in Panel C. The cells at a density of 1×10^5 cells/ml were treated with 15, 30, or 60 μmol/L EGCG or DMSO alone (0 μmol/L EGCG) as control for 8 h (A and C) or treated with 60 μmol/L EGCG for an indicated duration (B). Total cell lysates were subjected to western blot analysis with indicated antibodies.

Taken together, our data indicated that EGCG suppressed the cell proliferation of IMS-M2 cells through specific inhibited on NPM1 mutation.

3.3. EGCG suppressed AKT activity in IMS-M2 cells

It has been shown that constitutively active AKT protect cells from apoptosis. To clarify whether AKT were affected by EGCG in IMS-M2 cells, the activity of AKT in IMS-M2 cells treated with or without EGCG was meadured following the indicated duration as shown in Figure 3A. Western blot analysis using anti-phosphospecific-Akt antibody showed that EGCG suppressed AKT phosphorylation in a time dependent manner (Figure 3A). That means EGCG could induce cell death in IMS-M2 cells.

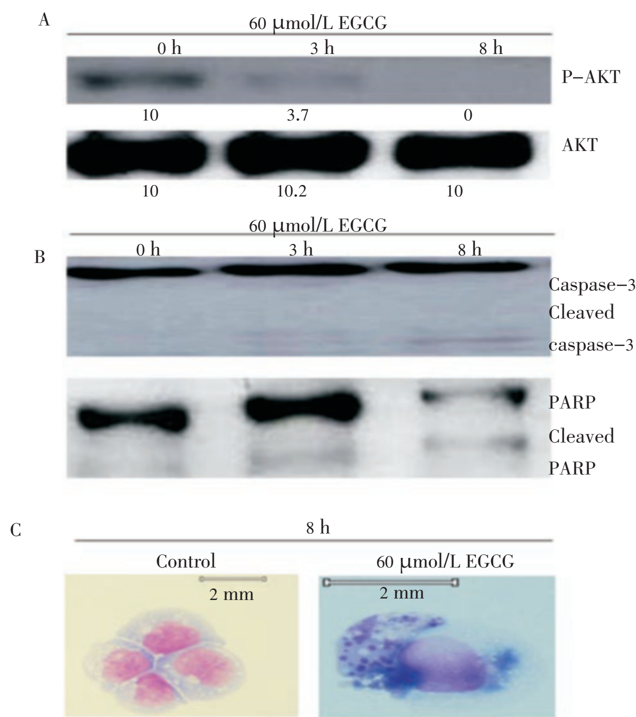

Figure 3. EGCG inhibited AKT activity and induce apoptosis in FLT3-mutated cells. Panel A showed the inhibitory effect of EGCG on AKT activity in IMS-M2 cells. IMS-M2 cells at a density of 1×10^5 cells/ml were treated with 60 μmol/L EGCG or DMSO alone (0 μmol/L EGCG) as control for an indicated duration. Total cell lysates were subjected to western blot analysis with indicated antibodies. Panel B and C showed the evidences of apoptosis induced by EGCG treatment in IMS-M2 cells. The activation forms of caspase-3 and inactivation form of PARP were detected after 8 h treated with 60 μmol/L EGCG (B). The morphology of IMS-M2 cells after treated with or without EGCG were evaluated. After 8 h treated with 60 μmol/L EGCG, cells were fixed onto slides and stained with Wright-Giemsa solution. The arrow indicated that the nuclei of IMS-M2 cells were fragmented by EGCG treatment (C).

3.4. EGCG induced apoptosis in IMS-M2 cells

AKT involved in apoptosis has been clarified. In these cells, AKT phoshorylation was inhibited by EGCG treatment (Figure 3A). To test whether EGCG induced apoptosis in IMS-M2 cells, the cell morphology and the status of some apoptotic markers in IMS-M2 cells were checked after the treatment with EGCG. As a result, after 8 h treatment with 60 μmol/L EGCG, cleaved caspase-3 were detected, then inactivated one enzyme involved in DNA repair, PARP were detected (Figure 3B). Caspase-3 proteolytic cleavage of PARP is a key event in apoptosis. In addition, the observed apoptotic bodies after 60 μmol/L EGCG treatment (Figure 3C) indicated that EGCG caused apoptosis in IMS-M2 cell line harboring *NPM1* mutations.

4. Discussion

As mentioned above, one of the potential strategies for treating AML patients with *NPM1* mutation is finding any reagents that can enhance the propensity of *NPM1*-mutated AML cells to die or to be killed[12]. In this study, we demonstrated that EGCG inhibited the cell proliferation and induced apoptosis in IMS-M2 cell line with *NPM1* mutation and suggested that EGCG could be a potential reagent for treating AML patients harboring *NPM1* mutation.

AML with mutant *NPM1* accounts for approximately one-third of all AMLs. Because of its distinctive molecular, pathologic, immunophenotypic and clinical characteristics [13,20], *NPM1*-mutated AML has been included as a provisional entity in the 2008 World Health Organization classification of lympho-hemopoietic neoplasms. Despite the advantages of understanding about the role of NPM1 in leukegenesis, the development of a targeted therapy for *NPM1*-mutated AML has still been a problem. For a long time, the predominant abnormal accumulation of NPM1 mutant in cytoplasm attracted the scientists' concern in finding drugs that can redirect NPM1 from cytoplasm to nucleus. However, it is very difficult to intervene on the abnormal traffic of the NPM1 mutant[20]. Leptomycin B is such a typical example, it can redirect NPM1 mutant to nucleoplasm but cannot direct to nucleolus (the physiological site of NPM1) [12]. Currently, Balusu *et al*. suggested another direction, that is, to interfere on the level or the oligomerization status of NPM1 that influence its capability to properly build up the nucleolus in *NPM1*-mutated AML cells[21]. Agreement with Balusu *et al*., we suggested that finding any reagents that can reduce or even completely inhibit the expression of NPM1 in AML with *NPM1* mutation leading to instability of nucleolus could be consider as potential strategies for treating AML with *NPM1* mutation.

EGCG, the major polyphenol of green tea, has been used as a beverage for over 5000 years. EGCG offers several potential clinical advantages compared to other traditional cancer drugs. Most modern medicines currently available for treating cancers are very expensive, while EGCG is globally available as tea, inexpensive to isolate and can be administered orally[22]. In addition, traditional cancer drugs that often destroy some healthy cells along with cancerous cells, while EGCG was noticed as an apoptosis inducer agent that is non-toxic to healthy cells[17,19,22,23]. Moreover, EGCG appears to target biochemical and genetic functions unique to cancer cells[17,22]. In this report we have shown that EGCG specifically targeted on NPM1 expression in IMS-M2 cells, but not in MOLM13 cells that carrying NPM1 wild-type. Some of the anti-carcinogenic agents currently in use have toxic adverse effects. However, data from clinical trials reported to date suggests that EGCG has a very acceptable safety profile[24]. It is noted that green tea is now developing as a cancer preventive drug in the USA and Europe[25]. Currently, there are 83 ongoing clinical trials studying the effects of EGCG on different pathologies[26]. These benefits support further development of EGCG as a potentially useful anti-carcinogenic agent.

AML patients harboring mutant *NPM1* often carry FLT3 mutations, particularly the *ITD*-type mutations and poor prognosis[14]. In another paper, we have also demonstrated that EGCG could down-regulate the expression of FLT3 in FLT3 mutated cell lines (not yet published data) suggesting that EGCG can be a potential reagent for treating AML patients harboring *NPM1/FLT3* mutation.

Conflict of interest statement

We declare no conflict of interest.

Acknowledgements

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Comments

Background

This is a good work and very informative that the author evaluated the down-regulated expression of NPM1 in IMS-M2 cell line by EGCG. Moreover, EGCG also suppressed the cell proliferation and induced apoptosis in IMS-M2 cells. In this study, the authors have suggested that EGCG could be considered as a reagent for treatment of AML patients with *NPM1* mutations.

Research frontiers

Studies are being performed in order to determine the effect of EGCG on Down-regulated expression of NPM1 in IMS-M2 Cell line.

Related reports

There are very limited reports on related to this study. But the authors have developed a good technique for a treatment of AML using EGCG.

Innovations and breakthroughs

In this study, the authors have evaluated the effect of EGCG for treatment of AML.

Applications

This study is very useful for treatment of AML patients.

Peer review

This is a good and useful study in which the authors have evaluated the down-regulated expression of NPM1 in IMS-M2 cell line by EGCG. The results obtained in this work clearly suggested that EGCG was good for the treatment of AML patients.

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