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THE POTENTIAL EFFECTS OF GREEN TEA (-)-EPIGALLOCATECHIN-3-GALLATE ON OVERCOMING IMATINIB-RESISTANCE IN CHRONIC MYELOID LEUKEMIA BEARING *BCR-ABL*

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ABSTRACT

We investigated the effect of (-)-epigallocatechin-3-gallate (EGCG) in overcoming imatinib mesylate -resistance in chronic myeloid leukaemia cells. Cell proliferation was determined by trypan blue dye exclusion test. Western blot analysis was performed to test the expression of key proteins. EGCG showed anti-proliferative effects in TCCY cells (IC_{50} = 23 μM), TCCY/T315I cells (IC_{50} = 19 μM) and wild type and mutant BCR-ABL-transfected Ba/F3 cells (IC_{50} from 30 to 33 μM). Moreover, treatment with EGCG (4 hours) resulted in decrease of BCR-ABL expression. Finally, EGCG treatment inhibited the phosphorylation of AKT and MAPK and induced apoptosis in these cells.

Keywords: BCR-ABL/T315I, CML, Imatinib-resistant, EGCG, apoptosis.

TÓM TẮT

Tiềm năng điều trị bệnh bạch cầu mạn dòng tuỷ mang tổ hợp gen BCR-ABL kháng Imatinib của tinh chất trà xanh Epigallocatechin-3-gallate

Trong nghiên cứu này chúng tôi nghiên cứu tác dụng của (-)-epigallocatechin-3-gallate (EGCG) trong việc điều trị bệnh bạch cầu mạn dòng tủy kháng imatinib mesylate. Sự tăng sinh tế bào được đánh giá bằng phương pháp nhuộm trypan blue. Kĩ thuật lai miễn dịch Western blot được dùng để kiểm tra sự biểu hiện của các protein mục tiêu. EGCG cho thấy có khả năng ức chế tăng sinh ở dòng tế bào TCCY (IC_{50} = 23 μM), TCCY/T315I (IC_{50} = 19 μM) và dòng tế bào Ba/F3 chuyển gen BCR-ABL. Ngoài ra, chúng tôi nhận thấy việc xử lí EGCG (4 giờ) đã làm giảm biểu hiện của protein BCR-ABL. Cuối cùng, xử lí EGCG ức chế hoạt tính của AKT và MAPK và cảm ứng gây chết apoptosis trong các dòng tế bào này

Từ khóa: BCR-ABL/T315I, CML, kháng Imatinib, EGCG, apoptosis.

1. Introduction

Patients with chronic myeloid leukaemia (CML) are commonly treated with a frontline-specific inhibitor of BCR-ABL tyrosine kinase inhibitor (TKI), imatinib mesylate (IM). IM inhibits kinase activities of BCR-ABL by inhibiting competitively the binding of

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ATP to its docking site [1,2]. However, approximately 95% of CML patients develop IM-resistance due to the acquired *BCR-ABL* gene mutation; which has emerged as a significant clinical problem [3-5]. IM strongly inhibit phosphorylation of tyrosine in wild type (WT) BCR-ABL whereas does not act on BCR-ABL with T315I mutations [6]. T315I mutation accounts for 15–20% of mutations of the ABL kinase domain, E255K and M351T mutations are also highly prevalent [7]. New TKIs including dasatinib and nilotinib overcame this problem to some extent but had no effect on the drug-resistant T315I mutation in CML patients [8]. Thus, it is urgent to develop more potent TKIs to circumvent the IM- resistance.

We previously reported that (-)-epigallocatechin-3-gallate (EGCG) can overcome IM resistance in gastrointestinal stromal tumour cells [9]. Therefore, in this study, we evaluated anticancer effects of EGCG in IM resistant CML cells. We investigated the growth inhibitory effects of EGCG on CML cell lines bearing wild type and mutant BCR-ABL and clarified possible mechanisms of those anticancer effects.

2. Materials And Methods

Cell lines, culture conditions

Experiments were conducted using two human leukaemia cell lines: TCCY (harbouring wild type *BCR-ABL*) and TCCY/T315I (harbouring T315I mutation in *BCR-ABL*) (kindly provided from Prof. Yuko Sato, Japan). The cells were grown in RPMI 1640 medium (Sigma-Aldrich, Ho Chi Minh, Viet Nam) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (JRH Biosciences, Lenexa, KS, USA m), 100 IU/ml penicillin, and 0.1 mg/ml streptomycin (Sigma-Aldrich, Ho Chi Minh, Viet Nam) in a humidified incubator of 5% CO_2 at 37^oC.

The parental Ba/F3 cells were cultured in RPMI 1640 medium (Sigma-Aldrich, Ho Chi Minh, Viet Nam) supplemented with 1 ng/ml interleukin-3 (IL-3, R&D Systems).

Plasmids constructs

Full-length human P210 BCR-ABL E255K cDNA (kindly provided by Dr. Charsle Sawyers U.C.L.A, USA), cloned into pMSCVpuro vector (Clontech, Laboratories, Inc, USA) at EcoRI sites, was re-cloned into the pcDNA3.1(+) vector, and was confirmed by sequencing. The pcDNA3.1–BCR-ABL/WT, pcDNA3.1–BCR-ABL/T315I and pcDNA3.1–BCR-ABL/Y253H vectors were created using the PrimeSTAR Mutagenesis Basal kit (Takara, Tokyo, Japan) according to the manufacturer's instructions. All constructs were verified by restriction enzyme digestion and DNA sequencing.

Generation of Ba/F3 cells expressing BCR-ABL/WT, BCR-ABL/T315I or BCR-ABL/Y253H

Ba/F3 cells stable expressing BCR-ABL/WT, BCR-ABL/T315I or BCR-ABL/Y253H were generated as described elsewhere [10]. Transformed Ba/F3 cells were maintained in RPMI 1640 medium containing 10% FBS in the absence of rmIL-3.

Reagents

EGCG was obtained and dissolved as described in detail previously [11].

Cell proliferation assays

The viability of cells was determined by trypan blue dye exclusion test as described previously [9]. 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 72 h. After treatment, 10 µl cell suspensions was mixed with 10 µl 0.4 % trypan blue, and viable cells were manually counted using a haemocytometer. Results were calculated as the percentage of values measured when cells were grown in the absence of reagents.

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 reagents. After incubation for indicated durations, cells were collected and washed twice with phosphate buffered saline (PBS) (−). Cells were then dissolved in a protein lysis buffer containing 5 mM ethylenediaminetetraacetic acid (EDTA), 50 mM NaF, 10 mM $Na_2H_2P_2O_7$, 0.01% Triton X-100, 5 mM N-2-hydroxyethyl piperazine-N′-2-ethanesulfonic acid (HEPES), 150 mM NaCl, 1 mM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride, and 75 µg/mL aprotinin on ice for 30 min with brief vortexing 4 times with every 10 min. After centrifugation at 13,000 rpm at 4°C for 10 min, total cell lysates were collected. Protein samples were electrophoresed through a polyacrylamide gel and transferred to a Hybond-P membrane (Amersham, Buckinghamshire, UK) by electro-blotting as described in detail previously [9]. After washing, the membrane was probed with antibodies, and antibody binding was detected using enhanced chemiluminescence ECL (Amersham). c- ABL, ERK1 (sc-93), total Akt (sc-1618), anti-rabbit IgG- HRP (sc-2317), and anti-mouse IgG-HRP (sc-2031) antibodies were obtained from Santa Cruz Biotechnology (Ho Chi Minh, Viet Nam). Anti-actin (A2066) antibody was from Sigma-Aldrich. Phospho-p44/42 Map kinase (Thr202/Tyr204) Phospho-Akt (Ser473), caspase-3 antibodies were from Cell Signaling Technology (Ho Chi Minh, Viet Nam). Anti-PARP antibody was from WAKO Chemicals (Osaka, Japan)

Statistical analysis

Values were expressed as the mean ± standard deviation. Statistical analyses were done using Student's *t-test*. $P < 0.05$ was considered to indicate a statistically significant difference.

3. Results

EGCG inhibited the growth of CML cells

To evaluate the effect of EGCG on cell growth, two cell lines [TCCY and TCCY/T315I] were incubated either with DMSO alone (0 µM EGCG) or with various concentrations of EGCG for 72 hours. The trypan blue exclusion test was used to assess cell proliferation. As we expected, EGCG showed growth inhibitory effect on both cell

lines. TCCY cells harbouring wild type *BCR-ABL* showed less sensitive to EGCG (IC_{50} = 23 μM) as compared with TCCY/T315I cells harbouring T315I mutation in *BCR-ABL* gene (IC_{50} = 19 μM) (Fig. 1B). However, the results of growth inhibitory effect of EGCG on Ba/F3 cells transfected with wild type (Ba/F3-WT) and mutant (Ba/F3-T315I and Ba/F3-Y253H) *BCR-ABL* (Fig. 1C) are different from that on human cells. The Ba/F3 cells harbouring wild type *BCR-ABL* (IC_{50} = 30 μM) seem to be more sensitive to EGCG than Ba/F3 cells harbouring T315I (IC_{50} = 33 μM) or Y253H mutation (IC_{50} = 32 μM).

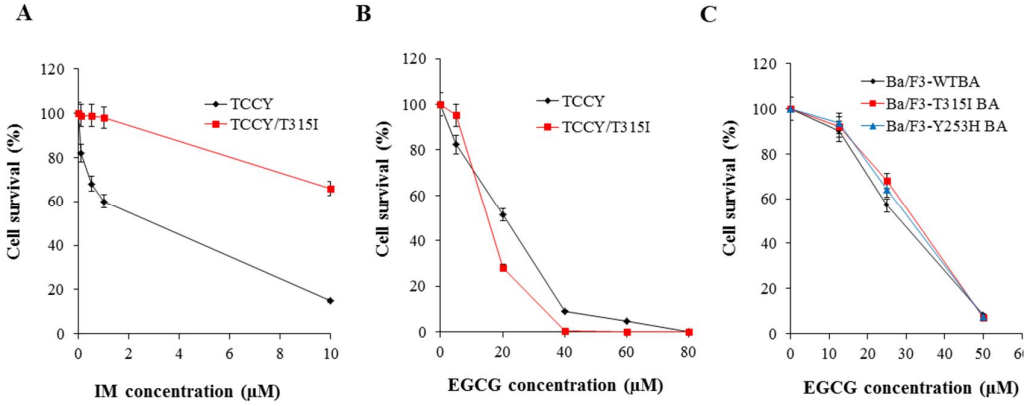

Figure 1. *EGCG inhibited the growth of CML cells*

TCCY, TCCY/T315I, Ba/F3–BCR-ABL/WT (Ba/F3-WTBA), Ba/F3–BCR-ABL/T315I (Ba/F3-T315I BA) and Ba/F3–BCR-ABL/Y253H (Ba/F3-Y253H BA) cells at a density of 1 x 10^5 cells/ml were treated with indicated concentration of IM (A) EGCG (B and C) or DMSO alone as control for 72 hours. The number of alive cells was counted after trypan blue exclusion test. Data were calculated as the percentage of the control values.

Decrease of BCR-ABL expression and phosphorylation of AKT and MAPK in EGCG-treated CML cells

It is well known that BCR-ABL plays an important role in pathogenesis of CML. Therefore, we analysed the expression of BCR-ABL in the presence of EGCG (60 μM) in TCCY and TCCY/T315I cells. Interestingly, the expression of BCR-ABL protein was significantly decreased after 4 hours exposure of TCCY or TCCY/T315I cells to 60 μM of EGCG (Fig. 2A). Moreover, EGCG also suppressed BCR-ABL expression in Ba/F3-WT, Ba/F3-T315I and Ba/F3-Y253H cells (Fig. 2B).

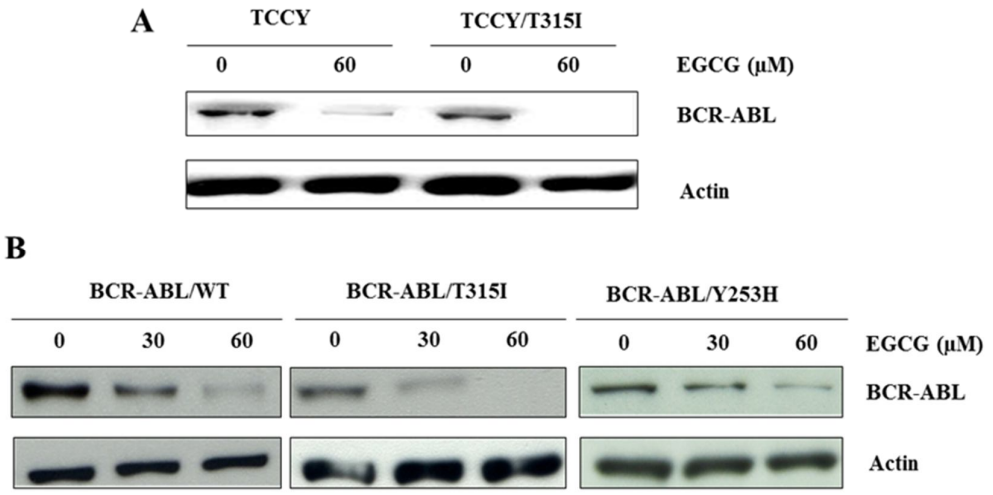

Figure 2. *Decrease of BCR-ABL expression in EGCG-treated CML cells*

BCR-ABL protein from *TCCY, TCCY/T315I (A) or Ba/F3–BCR-ABL/WT, Ba/F3–BCR-ABL/T315I and Ba/F3–BCR-ABL/Y253H (B) cells after 4 hours treatment. The cells at a density of 1 x* 10^5 *cells/ml were treated with indicated concentration of EGCG or DMSO alone as control . Total cell lysates were subjected to western blot analysis with indicated antibodies.*

Next, we measured the activity of MAPK and AKT in TCCY and TCCY/T315I cells after EGCG treatment. The phosphorylation of MAPK and AKT (p-MARK and p-AKT) were decreased in TCCY and TCCY/T315I cells after EGCG treatment in both dose and time dependent manner (Fig. 3 and 4). Notably, IM did not significantly inhibit the phosphorylation of AKT and MAPK in TCCY/T315I cells even at high concentration (up to 5 μM) (Fig. 4).

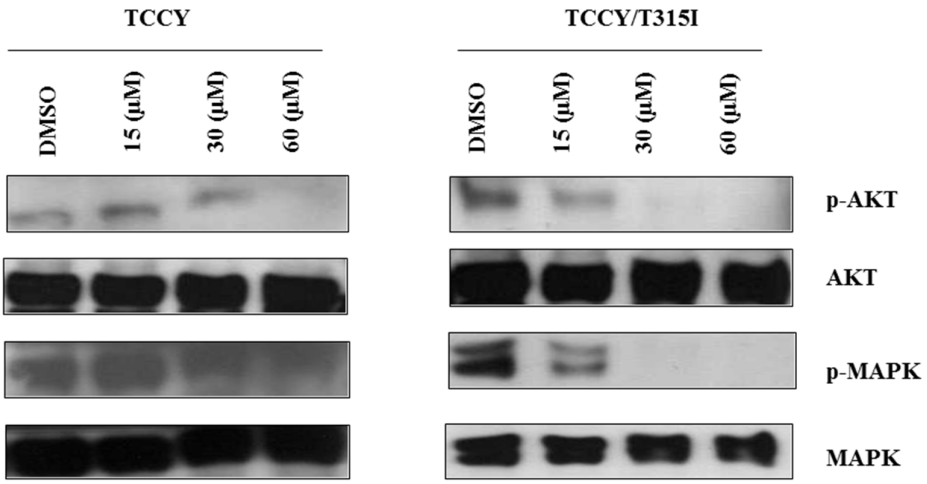

Figure 3. *EGCG inhibited the phosphorylation of AKT and MAPK in TCCY and TCCY/T315I cells in dose dependent manner*

AKT, MAPK, p-AKT, p-MAPK from TCCY and TCCY/T315I cells after treatment. The cells at a density of 1 x 10^5 cells/ml were treated with indicated concentration of EGCG or DMSO alone as control for 4 hours. Total cell lysates were subjected to western blot analysis with indicated antibodies.

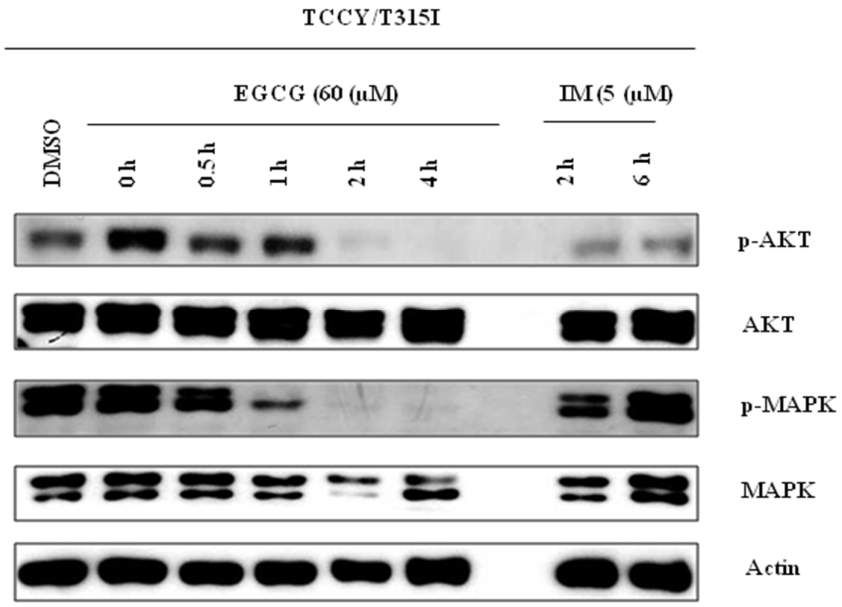

Figure 4. *EGCG inhibited the phosphorylation of AKT and MAPK in TCCY/T315I cells in time dependent manner*

AKT, MAPK, p-AKT, p-MAPK from TCCY/T315I cells after treatment. The cells at a density of 1 x 10^5 cells/ml were treated with 60 µM EGCG or 5 µM IM or DMSO alone as control for indicated hours. Total cell lysates were subjected to western blot analysis with indicated antibodies.

EGCG induced apoptosis in TCCY and TCCY/T315I cells.

To evaluate the effect of EGCG on apoptotic induction in TCCY and TCCY/T315I cell lines, cleaved PARP and cleaved Caspase-3 have been evaluated in TCCY and TCCY/T315I cells after EGCG treatment. Western blot analysis of these proteins showed that EGCG induced the cleavage of PARP and Caspase-3 (indicators of apoptosis) in these cell lines (Fig. 5). It demonstrates that EGCG triggers apoptosis in these cells.

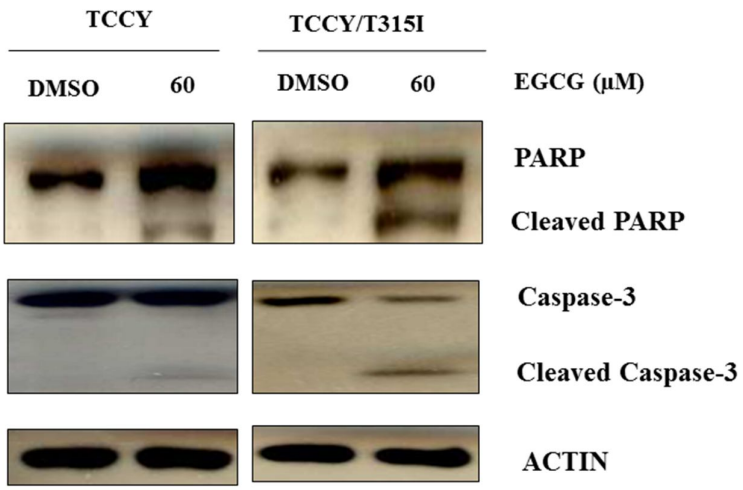

Figure 5. *EGCG induced cleavage of PARP and Caspase-3 in TCCY and TCCY/T315I cells*

(modified as previous photo) TCCY and TCCY/T315I cells at a density of 1 x 10^5 cells/ml were treated with 60 μM EGCG or DMSO alone as control for 8 hours. Total cell lysates were subjected to western blot analysis with indicated antibodies.

4. Discussion

Drug resistance during IM treatment is mostly related to the point mutations occurring within the kinase domain of BCR-ABL. Up to now, over 90 different point mutations in the BCR-ABL kinase domain have been identified from relapsed CML patients, who are resistant to IM. Most mutations, except T315I, may be eradicated with the appropriate choice and combinations of second generation TKIs. However, there are still no effective TKIs available for CML with the T315I mutation. Considering these facts, the T315I mutation remains a crucial clinical challenge, and it is imperative to develop novel strategies to overcome this resistance. The benefits of EGCG have been documented elsewhere [12]. Comparing to traditional cancer drugs which often causes side effects by not recognizing healthy cells and cancer cells to target [13], EGCG has been demonstrated to only target on cancer cells with an acceptable safety profile [14]. These benefits further support for the development of EGCG as an anti-cancer agent. The principal objective of this study is to identify the effective of EGCG against CML cells, especially in cells carrying T315I mutation in *BCR-ABL*. We demonstrated that EGCG had **growth** inhibitory effects on cells that carrying wild type as well as mutant BCR-ABL. In human cells, IM resistant TCCY/T315I (IC_{50} = 19 μM) cells showed more sensitive to EGCG as compared to IM sensitive TCCY cells (IC_{50} = 23 μM) (Figure 1A). However, the effects are not the same when EGCG was tested in *BCR-ABL*-transfected Ba/F3 cells (Figure 1B). It seems that there is no difference in action of EGCG on all types of *BCR-ABL* transfected Ba/F3 cells. In this report, EGCG showed different **growth** inhibitory effects on human and transfected cells. However, the mechanism of these differences are not understood.

As demonstrated in this study, EGCG treatment could affect on the phosphorylation of AKT and MAPK (Figure 3 and 4). These molecules have been considered as downstream effectors of BCR-ABL [15]. The inhibition of AKT and MAPK phosphorylation caused the cell death in TCCY and TCCY/T315I by inducing cleaved PARP and cleaved caspase-3 (Figure 5). Interestingly, EGCG treatment after 4 hours could results in the decrease of BCR-ABL expression. The decrease of BCR-ABL could be the main factor triggering anticancer effect of EGCG on CML cells. However, the mechanisms of EGCG-suppressed BCR-ABL expression are not clarified yet and further studies need to be conducted.

5. Conclusion

Our results could suggest further studies to investigate the potential use of EGCG in order to overcome IM resistance.

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