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ORIGINAL ARTICLE

Effects of green tea epigallocatechin-3-gallate on transcription factors regulating expression of FLT3

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Summary

Purpose: *In our previous paper we previously reported that epigallocatechin-3-gallate (EGCG) inhibits FLT3 expression in cell lines harboring FLT3 mutations. In this research, we carried on to investigate the influence of EGCG on FLT3 promoter activity and FLT3 transcription.*

Methods: *The effect of EGCG on the mRNA expression of flt3 and flt3-promoter activity was evaluated using semi-quantitative reverse transcription-PCR and luciferase reporter assay. The gene expression profiling analysis was done for detecting the effect of EGCG on flt3-transcription factors. Then, the protein level of C-MyB was obsvered using western blot analysis.*

Results: *The results showed that EGCG reduced the transcription level of FLT3 by suppressing its promoter activity. By doing gene expression profile analysis in MOLM-13 cells established from acute monocytic leukemia patient with two mutations within FLT3 EXON 14 in a time-dependent manner, we found that the expression of mRNA of FLT3 was first observed to downregulate at 6 h together with the decreasing of Homeobox A9 (HOXA9) transcription factor after EGCG treatment. The changing of C/EBPa expression was found at 8 h. Interestingly, the reducing mRNA of c-Myb by EGCG was observed at 4 h, earlier than FLT3 was downregulated. There was no change in Meis Homeobox 1 (Meis1) by EGCG. We also found the protein level c-Myb was inhibited by EGCG in MOLM-13 and MOLM-14 cells after treating these cells with 60μM of EGCG for 8 h.*

Conclusion: *This data indicated the involvement of transcription factors in controlling the expression of FLT3 by EGCG.*

Key words: *EGCG, Hoxa9, MEIS1, C-Myb, transcription factor, FLT3*

Introduction

The transcription of *FLT3* was reported to be regulated by Homeobox A9 (*HoxA9*), Meis Homeobox 1 (*Meis1*), cellular oncogene Myb (*c-Myb*) and CCATT/Enhancer binding protein alpha (*C/EBPa*) [1-3]. *HoxA9* is expressed at high levels in early hematopoietic progenitor cells and promotes stem cell expansion. In contrast, *Hoxa9* downregulation is associated with hematopoietic differentiation [4,5]. The *Hox* cofactor- *Meis1* cooperates with *Hox* genes to accelerate the onset of acute myeloid leukemia (AML) in mouse models [6]. Interestingly, *Meis1* is frequently found upregulated along with *Hox* genes in human leukemias and this is associated with particularly high levels of *FLT3* mRNA [7]. Chromatin immunoprecipitation has confirmed co-occupancy of *Hoxa9* and *Meis1* on the *FLT3* promoter in myeloid leukemogenesis and lympho-hematopoietic models [1-3]. Knockdown of *Hoxa9* significantly reduced *FLT3* transcription and expression [1]. Conversely, forced expression of *Hoxa9* increased *FLT3* transcription and expression in a Pro-B cell line that expressed low levels of *FLT3* [1].

So far, a few hints of a connection between *FLT3* and CCATT/enhancer binding protein alpha

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(*C/EBPa*) markers have been given by recent studies showing that the favorable outcome associated with *C/EBPa* bi-allelic mutations was cancelled out by the presence of *FLT3*-ITD mutations [8,9]. *FLT3*-ITD signaling inhibits *C/EBPa* differentiating function by promoting its phosphorylation [10], and *C/EBPa* with a C-terminal mutation collaborates with *FLT3*-ITD in inducing AML [11]. Such collaborations may rely on the ability of *FLT3* signaling to support myeloid commitment of the expanding *C/EBPa* mutated cells [8]. A study on the profiling arrays from AML patients with differing *C/EBPa* status links *FLT3* expression and *C/EBPa* activity has shown that *C/EBPa* bi-allelic mutations associate with lower levels of *FLT3* transcript in AML patients whereas no significant differences were observed in *FLT3* expression between patients with wild type or mono-allelic *C/EBPa* mutations [3].

Notably, *c-Myb* was identified as an important downstream target of *Hoxa9* and Meis [12]. The cooperative activities of *c-Myb* and *C/EBPa* in activating the promoter of myeloid genes including *FLT3* have been previously reported [3,13,14]. Together with *Hoxa9* and *Meis1*, *C/EBPa* and *c-Myb* are important elements of the combinatorial binding of leukaemia-related transcription factors that regulate *FLT3* expression [3].

Our previous paper [15] reported that (-)-epigallocatechin-3-gallate (EGCG) inhibits *FLT3* expression in cell lines harboring *FLT3* mutations. In this research, we continued to investigate the influence of EGCG on *FLT3* promoter activity and *FLT3* transcription.

Methods

Cell lines and culture conditions

The experiments were conducted using human leukemia cell lines with *FLT3* expression including MOLM-13 and MOLM-14 [2]. IMS-M2 cell line with no-expression of *FLT3* was used as a control in gene expression profiling assay [16]. The above cells were grown in Roswell Park Memorial Institute (RPMI) 1640 medium (Sigma-Aldrich, TBR Technology Corporation, Hochiminh City, Vietnam) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Thermofisher Scientific, Hochiminh City, Vietnam), 100 IU/ml penicillin, and 0.1 mg/ml streptomycin (P4333, Sigma-Aldrich, Hochiminh City, Vietnam) in a humidified incubator of 5% CO_2 at 37°C.

The 293T cells were grown in Dulbecco's modification of Eagle's medium (DMEM) (Sigma-Aldrich, Hochiminh City, Vietnam) supplemented with 10% FBS, 100 IU/ml penicillin, 0.1 mg/ml streptomycin, 1% sodium pyruvate and 1% L-glutamine (Biobasic, TBR Technology Corporation, Hochiminh City, Vietnam) in a humidified incubator of 5% CO_2 at 37°C.

Reagents

EGCG generously gifted by Dr. Yukihiko Hara (Japan) was dissolved in dimethylsulfoxide (DMSO) (Sigma-Aldrich, TBR Technology Corporation, Hochiminh City, Vietnam). 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.

Plasmids constructs

The *FLT3* promoter was amplified by using the following primers: forward primer: 5'-TAATCTCGAGCGTGGAATTCCTAGAATTGG-3' and reverse primer 5'- ACGCAAGCTTGGCCTCCGGAGCCCGGGGT-3'. The amplified DNA fragments were digested with XhoI and HindIII and inserted into the XhoI/HindIII site of the pGL4-10 plasmid (Promega, Hochiminh City, Vietnam).

All the constructs were verified by restriction enzyme digestion and DNA sequencing.

Luciferase reporter assay

To check *FLT3* promoter activity, each reporter plasmid (100ng) and 5 ng of a renilla-luciferase plasmid (as an internal control) were transfected into 293T cells (1×10^5 cells per well in 48-well plates) by Lipofectamine, following the manufacturer's instructions. Luciferase activity was measured with the Dual-Luciferase Re-porter Assay System (Promega, Tokyo, Japan). Promoter activities were expressed as the ratio of *Firefly* luciferase to *Renilla* luciferase activities.

Semiquantitative reverse transcription-PCR

Total RNA was extracted from the cells treated with or without 60μM EGCG for 8 h by using Sepasol (Nakalai). First-strand cDNA was synthesized from 1 μg of total RNA using SuperScript II Reverse Transcriptase (Invitrogen, Carlbad, CA, USA) and random hexamer. A segment of *FLT3* spanning exon 14 and 15 was amplified by using primers *FLT3*.1675F (5'-GACAACATCTCATTCTATGCAAC-3') and *FLT3*.18R1 (5'-TCTGAACTTCTCTTGAACCA-3'). The thermal cycling profile was: 94°C for 2 min, followed by 40 cycles of 94°C for 30s, 55°C for 30s, 72°C for 1 min and a final extension at 72°C for 5 min. PCR products were separated by electrophoresis on a 1.5% agarose gel.

Gene expression profiling analysis

MOLM-13 and IMS-M2 cells were treated with or without 60μM EGCG for 2, 4, 6, 8 and 12 h. The mRNA was extracted (Trizol reagent; Invitrogen), following the manufacturer's instructions. cDNA was synthesized from the total RNA using a Gene Chip Expression 3'-Amplification Reagents One-Cycle cDNA Synthesis Kit (Affymetrix, Santa Clara, CA, USA). The DNA microarray analysis was performed as described previously using Affymetrix Gene Chip technology [17] by Dr. Kazuki Yasuda (National Center for Global Health and Medicine, Tokyo, Japan).

Western blot analysis

Cells were plated onto 6-well plates at a density of 1×10^5 cells/ml in the presence of EGCG. After incubation at indicated durations, cells were collected and washed twice with phosphate buffered saline (PBS) (-). Cells were then dissolved in a protein lysis buffer and western blot analysis was performed as previously described [15]. The FLT-3/FLK-2 (S-18) (sc-480) antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA), anti-actin (A2066) was from Sigma-Aldrich. Normal rabbit IgG (# 2729S) and *c-Myb* (D2R4Y) antibodies were from Cell Signaling Technology Japan (Tokyo, Japan).

Statistics

All data is expressed as the mean±standard deviation from three independent experiments or more. Statistical analysis was performed using an unpaired two-tailed Student's t-test for comparison of two groups or a two-way non-repeated variance measurement analysis followed by a Bonferroni *post-hoc* test for comparison of multiple groups. Such statistical analyses were carried out using microsoft excel 2010 for MAC (Microsoft Corporation, Redmond, WA, USA) with the add-in program excel statistical program file YSTAT2008 (Igakutosho-Shuppan, Ltd., Tokyo, Japan). P<0.01 was considered to reflect statistically significant differences.

Results and Discussion

Suppression of FLT3 gene expression by EGCG

To examine whether the downregulation of *FLT3* protein by EGCG [15] resulted partly from the reduction of its transcriptional level, we evaluated the effects of EGCG on transcriptional regulation of *FLT3* in MOLM-13 and MOLM-14 cells

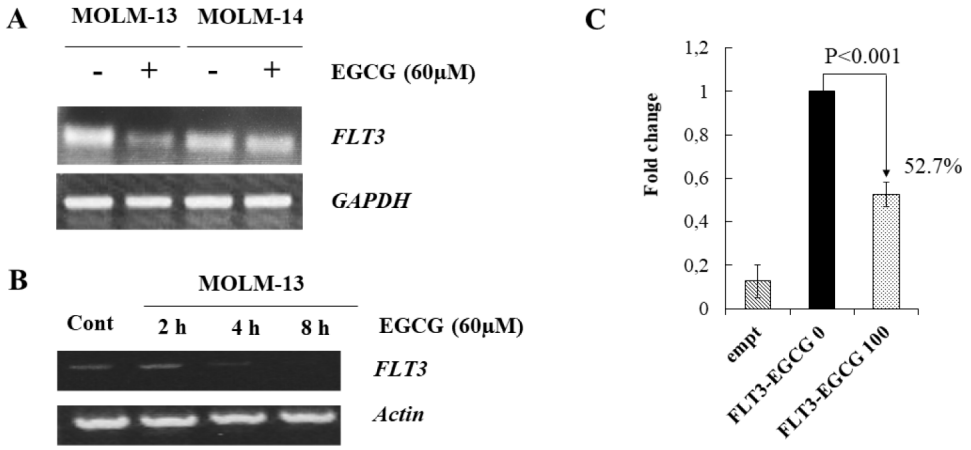

Figure 1. Western blot shows that EGCG inhibited *FLT3* promoter activity. **A, B:** Semi-quantitative reverse transcription-PCR, MOLM-13 and MOLM-14 cells at a density of 1×10^5 cells/ml treated with 60 μM EGCG for an indicated duration. Total RNA was extracted and cDNA was synthesized to perform PCR reaction with indicated primers. **C:** The inhibition of EGCG on *FLT3* promoter activity was analyzed by reporter assay (n=5, mean±SD). Luciferase activities of reporter series were tested in the presence or absence of 100 μM EGCG.

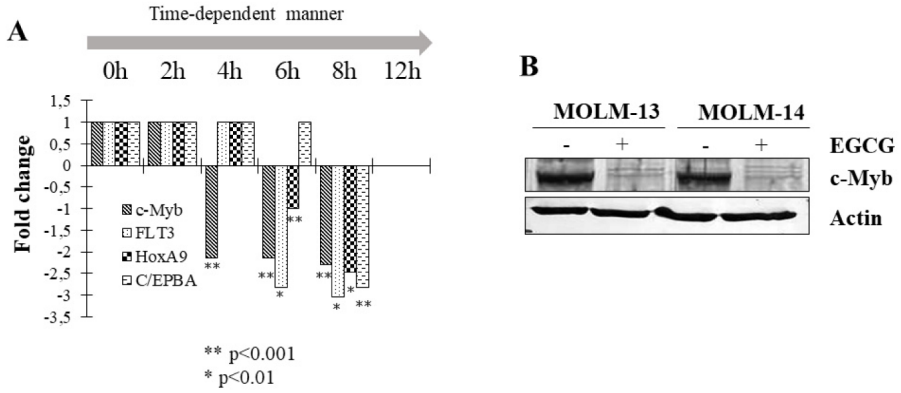

Figure 2. EGCG inhibited *FLT3* transcriptional factors. **A:** The fold change of mRNA of *c-Myb, FLT3, HoxA9* and *C/EPBa* in MOLM-13 cells after treatment with 60μM EGCG for the indicated hours by gene expression profile analysis. **B:** MOLM-13 and MOLM-14 cells at a density of 1×10^5 cells/ml were treated with indicated concentration of 60μM EGCC or DMSO alone as control for 8 h. Total cell lysates were subjected to western blot analysis with c-Myb and Actin antibodies. The Figure shows that the protein level c-Myb was inhibited by EGCG in MOLM-13 and MOLM-14 cells after treatment of these cells with EGCG.

with or without EGCG (60 μM) treatment for 8 h. The results in Figure 1A indicated that mRNA of *FLT3* was significantly reduced after treated with EGCG in MOLM-13 and MOLM-14 cells. Moreover, the mRNA level of *FLT3* also decreased in a time-dependent manner in MOLM-13 cells (Figure 1B)

A transient transfection study using the *FLT3* promoter-reporter construct was performed. A portion of the 5'-flanking region of the *FLT3* gene has been cloned. As shown in Figure 1C, EGCG significantly inhibited *FLT3* promoter activity.

EGCG inhibited FLT3 transcriptional factors

Hoxa9, *Meis1*, *C/EBPa* and *c-Myb* are important elements of the combinatorial binding of leukaemia-related transcription factors that regulate *FLT3* expression [16]. In order to evaluate the effect of EGCG on these factors, the mRNA level of MOLM-13 cells after treatment with or without EGCG for 2, 4, 6, 8 and 12 h was extracted and the gene expression profile analysis was done. As shown in Figure 2A, downregulated expression of mRNA of *FLT3* was observed at 6 h together with decrease of *Hoxa9* after treatment with EGCG. The changing of *C/EBPa* expression was found at 8 h whilst no changing in *Meis1* by EGCG was observed.

Interestingly,the lessening of transcription of *C-Myb* was first observed after 4 h treatment with EGCG, whereas the reduction of transcription of *FLT3*, *HOXA9* and *C/EBPA* were first detected after 6 h incubation with EGCG (Figure 2A). Notably, the promoter of the *FLT3* gene in our experiment contained many sites for *c-Myb* binding (-797, -773, -745, -713, -443, -327 and-274, by TFSEARCH and PROMO software). Moreover, the protein level *c-Myb* was inhibited by EGCG in MOLM-13 and MOLM-14 cells after treating these cells with 60μM of EGCG for 8 h as shown in Figure 2B. From this interesting initiating data, more experiments need to be done to investigate the role of EGCG in the regulation of *c-Myb*, as well as more reliable data about whether the recruitment of other transcriptional factors to *FLT3* promoter could be effected by EGCG.

Conclusion

In the present study, we noted evidence that EGCG suppressed *FLT3* promoter activity and its transcription. Taken together with our previous report [2], EGCG is an effective inhibitor of *FLT3* at both transcription and protein levels.

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Conflict of interests

The authors declare no conflict of interests.

References

1. Abramovich C, Humphries RK. Hox regulation of normal and leukemic hematopoietic stem cells. Curr Opin Hematol 2005; 12:210-6.
2. Bui TKL, Hoang TC, Yamagishi M et al. Inhibition of FLT3 expression by green tea catechins in FLT3 mutated-AML cells. PLoS One 2013;8:e66378.
3. Burk O, Mink S, Ringwald M, Klempnauer KH. Synergistic activation of the chicken mim-1 gene by v-myb and C/EBP transcription factors. EMBO J 1993;12:2027-38.
4. Gwin K, Frank E, Bossou A, Medina KL. Hoxa9 regulates Flt3 in lymphohematopoietic progenitors. J Immunol 2010;185:6572-83.
5. Hess JL, Bittner CB, Zeisig DT et al. c-Myb is an essential downstream target for homeobox-mediated transformation of hematopoietic cells. Blood 2006;108:297-304.
6. Hoang TC, Hoang AV, Iwasaki R et al. Detection of exon 12 type A mutation of NPM1 gene in IMS-M2 cell line. Leuk Res 2009;34:261-2.
7. Kato N, Kitaura J, Doki N et al. Two types of C/EBPalpha mutations play distinct but collaborative roles in leukemogenesis: lessons from clinical data and BMT models. Blood 2011;117:221-33.
8. Oelgeschlager M, Nuchprayoon I, Luscher B, Friedman AD. C/EBP, c-Myb, and PU.1 cooperate to regulate the neutrophil elastase promoter. Mol Cell Biol 1996; 16: 4717-4725.

9. Pineault N, Helgason CD, Lawrence HJ, Rk H. Differential expression of Hox, Meis1, and Pbx1 genes in primitive cells throughout murine hematopoietic ontogeny. Exp Hematol 2002;30:49-57.
10. Radomska HS, Alberich-Jorda M, Will B, Gonzalez D, Delwel R, Tenen DG. Targeting CDK1 promotes FLT3-activated acute myeloid leukemia differentiation through C/EBPalpha. J Clin Invest 2012;122:2955-66.
11. Reckzeh K, Bereshchenko O, Mead A et al. Molecular and cellular effects of oncogene cooperation in a genetically accurate AML mouse model. Leukemia 2012;26:1527-36.
12. Renneville A, Boissel N, Gachard N et al. The favorable impact of CEBPA mutations in patients with acute myeloid leukemia is only observed in the absence of associated cytogenetic abnormalities and FLT3 internal duplication. Blood 2009;113:5090-3.
13. Roche J, Zeng C, Baron A et al. Hox expression in AML identifies a distinct subset of patients with intermediate cytogenetics. Leukemia 2004;18:1059-63.
14. Thorsteinsdottir U, Mamo A, Kroon E et al. Overexpression of the myeloid leukemia-associated Hoxa9 gene in bone marrow cells induces stem cell expansion. Blood 2002;99:121-9.
15. Tsuchiya M, Tsuchiya K, Yasuda K et al. MafA is a Key Molecule in Glucose and Energy Balance in the Central Nervous System and Peripheral Organs. Int J Biomed Sci 2011;7:19-26.
16. Volpe G, Walton DS, Del-Pozzo W et al. C/EBPalpha and MYB regulate FLT3 expression in AML. Leukemia 2013;27:1487-96.
17. Wang GG, Pasillas MP, Kamps MP. Persistent transactivation by meis1 replaces hox function in myeloid leukemogenesis models: evidence for co-occupancy of meis1-pbx and hox-pbx complexes on promoters of leukemia-associated genes. Mol Cell Biol 2006;26:3902-16.