# Investigation of the Association Between TET2 Expression and Response to CAPE

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**ABSTRACT**

Acute myeloid leukemia (AML) is a clonal, neoplastic disease characterized by abnormal proliferation of myeloid progenitor cells. Genetic and epigenetic changes in AML patients impair proliferation and differentiation of myeloid progenitor cells. TET2 functions in DNA demethylation and mutations are frequently observed in patients with AML. Caffeic acid phenethyl ester (CAPE) is an active component of propolis, a resinous substance collected by honey bees from various plant sources. CAPE's antioxidant, anti-inflammatory, antiviral, immunostimulant and anticancer effects have been shown in various studies. In this study, we aimed to investigate CAPE's effect on TET2 mRNA expression. In K562 cell line, TET2 mRNA expression upon CAPE treatment was controlled with qRT-PCR. To decrease TET2 mRNA expression commercially available TET2 mRNA targeting shRNAs and a control shRNA were used. Transfection grade plasmids were then used to transfect K562 cells and antibiotic selection was applied for stable transfection. CAPE activity on cell viability in TET2 downregulated cells was assessed with Wst-1 assay. 5μM CAPE treatment increased TET2 mRNA expression fold change up to 2.5 times ($p< 0.05$). Two different shRNAs downregulated TET2 mRNA expression almost 50% compare to control plasmid ($p< 0.05$). TET2 downregulated cells were found more resistant to CAPE treatment (shRNA1; $p< 0.0001$ and shRNA2; $p< 0.05$). Our findings suggest that CAPE acts on cells by changing TET2 mRNA expression in K562 leukemia cells. In addition, TET2 downregulated cells were found more resistant to CAPE suggesting that TET2 expression may play a role in response to conventional treatments.

**Keywords:** Leukemia, TET2 expression, shRNA-mediated gene silencing, Anti-cancer

## INTRODUCTION

Acute Myeloid Leukemia (AML) is a clonal, heterogeneous, neoplastic disease characterized by abnormal proliferation of myeloid progenitor cells.[1] Genetic and epigenetic changes (DNA methylation and histone methylation/ acetylation) impair proliferation and differentiation of progenitor cells.[2] Blast cells lose their plasticity while continue to divide and proliferate. In recent years, with the conventional therapeutics, remission rate is up to 70 % in younger patients while it is 20-30% in elderly patients.[3]

Propolis is a bee product and used for disinfecting and repairing hives by bees. Chemical compound consist of phenolic acids, phenolic acid esters, flavonoids, cinnamic acid, coumaric acid, terpenes, hesperidin, nicotinic acid, caffeic acid and caffeic acid phenethyl ester (CAPE).[4]

CAPE is one of the active compounds of propolis and it has analgesic, anti-inflammatory, anti-fungal, anti-viral, anti-oxidant and anti-cancer properties have been investigated in various in vitro and in vivo studies.[5-8] CAPE has been shown to induce apoptosis in different cancer cell lines.[9-12]

However, the molecular mechanisms of how CAPE inhibits proliferation and induces apoptosis are not well understood. CAPE has been shown to downregulate NFkB expression which is overexpressed in many cancer cells.[13] Therefore, it is suggested that CAPE could induce apoptosis via NFkB pathway, however, it has not been validated in many cancer cells.[14,15] In addition, CAPE is suggested to have selective anti-tumoral property and increase cancer cell sensitivity to chemotherapy[16] and radiotherapy.[17] On the other side, the effect of CAPE in different cellular processes in cancer cells remains to be elucidated.

TET2 belongs to Ten Eleven Translocation (TET) gene family and encodes 2-oxoglutarate and Fe (II) dependent hydroxylases. TET2 is expressed in kidney cells, brain cells, hematopoietic system and has two isoforms.[18] TET enzymes are responsible in hydroxymethylcytosine (5-hmC) production. Cytosine nucleotide is methylated (5-mC) as an important epigenetic modification in cells. TET enzymes catalyze oxidation of 5-methyl groups, create 5hmC and initiates a process called DNA demethylation. 5-hmC restricts binding of many methyl-CpG-binding proteins to DNA and inhibits DNA methylation (5-mC production).[19] Various mutations have been identified on TET2 gene in various myeloid and lymphoid malignancies.[20] Insertions, deletions, nonsense mutations and frameshift mutations on the gene impair protein function. Studies with TET2 knockout mice showed that amount of genomic 5hmC decreased and developed myeloid malignancies.[21] In addition, shRNA mediated silencing of TET2 in CD34+ human umbilical cord blood cells decreased 5-hmC level and increased monocyte cells.[22] Moreover, TET2 mutations have been associated with poor prognosis in AML subgroups.[23] These results indicate that disruption of TET2 function could have an important role in the development of myeloid malignancies.

Impact of TET2 mutations in cancer cell response to current therapeutics including DNA hypomethylating agents has been studied in leukemia.[24] However, there is little information about how anti-cancer drugs effects TET2 expression in leukemia. It is still a question whether TET2 expression affects cellular response to chemotherapeutics.

In this study we aimed to investigate whether CAPE has an effect on TET2 expression in leukemia. For this aim, we used K562 cell line as it does not have any mutation on TET2 gene according to Cancer Cell Line Encyclopedia (CCLE).

## MATERIALS AND METHODS

### *Cell Culture and CAPE Treatment*

K562 cells were cultured in RPMI 1640 medium complemented with 10% FBS, 1% pen/strep and 1% l-glutamine. Cells were grown at 37$^0$C with 5% CO2 in atmospheric oxygen.

CAPE dissolved in DMSO to obtain 1mM solution and added to cell culture medium to obtain 5 μM final concentration. For control, same amount of DMSO added.

### *RNA Isolation and cDNA Synthesis*

To investigate TET2 mRNA expression upon CAPE treatment and quantify TET2 downregulation by shRNAs qRT-PCR was applied. RNA was isolated from cells using Nucleospin RNA isolation kit according to manufacturer's instruction. 1 μg RNA was used for reverse transcription using Luna cDNA synthesis kit.

### *qRT-PCR*

To analyze TET2 mRNA expression following primers were used. qRT-PCR reactions were carried on RotorGene (Qiagen) instrument using SYBR Green PCR Master Mix (Applied Biosystems). Ct results were normalized to RPLP0 housekeeping gene. Relative mRNA fold changes were calculated using ΔΔCt method.

### *shRNA Mediated Downregulation of TET2 Expression*

To evaluate a possible association between TET2 expression and response to CAPE treatment, TET2 mRNA expression was downregulated in K562 cells by using suresilencing shRNA plasmids. Two different shRNA against TET2 and one negative control shRNA sequences designed by the manufacturer and cloned in puromycin-resistant plas-

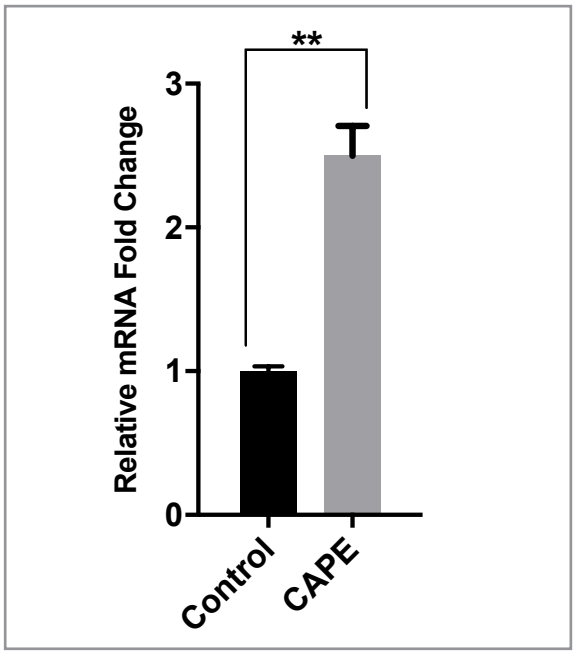


**Figure 1.** CAPE effect on TET2 mRNA expression. 5μM CAPE treatment increased TET2 mRNA expression nearly 2.5 fold. Untreated control group accepted as 1 fold and CAPE treated compared to control. Data presented as mean ±SD, **p< 0.01.

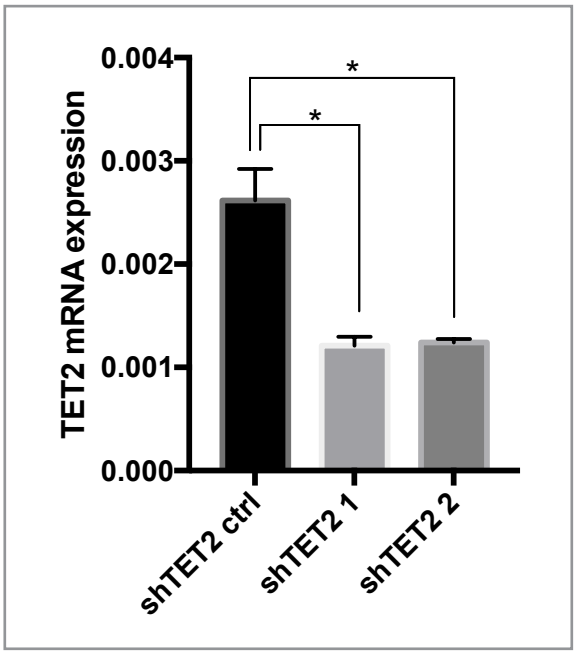


**Figure 2.** shRNA mediated downregulation of TET2 mRNA expression. 2 different shRNA decreased mRNA expression to half compared to control plasmid. Data presented as mean ±SD, *p< 0.05.

mid. Total three different plasmids individually prepared as transfection grade by transforming into DH5α competent bacteria. K562 cells transfected with these plasmids using Fugene HD transfection reagent according to shRNA plasmid manufacturer with a slight difference. Briefly 5 x $10^4$ cells plated into each 5 wells of a 24 well plate and incubated for 3 hrs. Then cells transfected with 0.5 μg plasmid and transfection reagent (1: 3 ratio) and removed back to incubator. Following day medium changed. At day 2 medium changed and antibiotic selection started. Cells treated with puromycin for 3 days. After 3 days medium replaced. Downregulation of TET2 validated with qRT-PCR.

### *Wst-1 Cell Viability Assay*

K562 control and TET2 downregulated cells were plated in a 96 well plate at a 25000 cells/ml concentration. Cells cultured in medium either containing 5 μM CAPE or DMSO for 3 days. End of the experiment, wst-1 solution was added into each well at 1:10 final dilution and plate was incubated at $37^0$C in 5% $CO_2$ for additional 4 hours. After the incubation OD values was recorded at 450nm on a plate reader. Cell viability was calculated as % and CAPE treated cells compared to DMSO treated cells (100%).

### Statistical Analysis

Cell viability assay was performed in quintet wells. qRT-PCR was performed in duplicates. All experiments repeated three times except from qRT-PCR, which was repeated two times. Data analyzed and graphs were prepared in GraphPad Prism V7 software. Student's t test with Welch's correction was applied to compare fold changes.

## RESULTS

### *CAPE Increase TET2 mRNA Expression*

In our previous study, we found that CAPE kills almost 50 % of K562 cells. Thus, in this study, K562 cells were treated with 5 μM CAPE for 3 days. Then, cells were collected and RNA was isolated. TET2 mRNA expression was evaluated with qRT-PCR. We found that 5 μM CAPE treatment increased TET2 mRNA expression fold change up to 2.5 times (p< 0.05) (Figure 1).

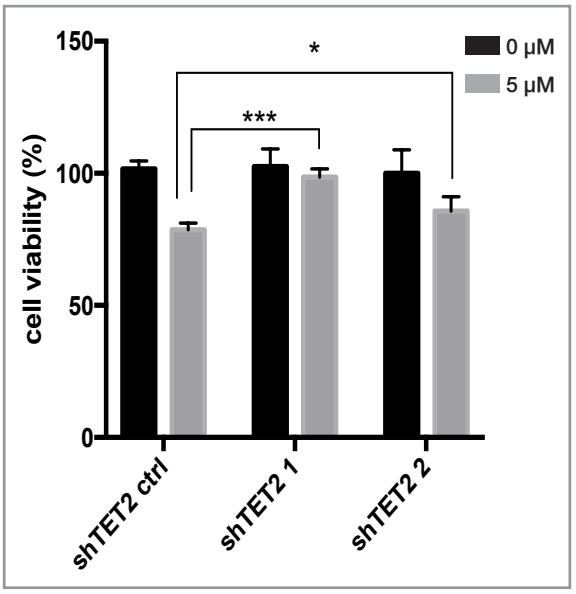


**Figure 3.** Cell viability results of CAPE treated TET2 downregulated cells. Cells treated with 5 µM CAPE for three days. Cell viability was assessed with Wst-1 assay. shRNA1 and 2 od values compared to shRNA control in percentage. K562 cells were found more resistant to CAPE upon TET2 downregulation. Data presented as mean ± SD, *p< 0.05, *** p< 0.001.

### *TET2 shRNA-Mediated Downregulation*

As CAPE treatment increased TET2 mRNA expression in K562 cells, we questioned if TET2 silencing changes cellular response to CAPE treatment. With this aim, we used commercially available shRNA plasmid system. Two different shRNAs downregulated TET2 mRNA expression almost 50% compare to control plasmid ($p< 0.05$) (Figure 2). TET2 downregulation did not change cell proliferation substantially.

### *TET2 Downregulated Cells Resistant to CAPE Treatment*

K562 cells transfected with two different TET2 shRNA and a negative control shRNA were treated with 5 µM CAPE for 3 days. At the end of experiment, cell viability was assessed using WST-1 assay. Absorbance values of shRNA1 and shRNA2 transfected cells were compared to control plasmid transfected untreated cells in percentage (Figure 3). TET2 downregulated K562 cells were found more resistant to 5 µM CAPE treatment compare to control cells according to cell viability assay results (shRNA1; $p< 0.0001$ and shRNA2; $p< 0.05$).

## DISCUSSION

CAPE is a lipophilic derivative of caffeic acid and an active component of propolis. It has a strong phenolic anti-oxidant property.[25] In addition, CAPE's anti-cancer effect has been shown to inhibit cell proliferation in various cancer types including breast, prostate, lung, colon, cervix.[7] Normal healthy cells have been shown to be more resistant to CAPE treatment compare to cancer cells suggesting that CAPE might have cancer-specific therapeutic property.[26] Moreover, CAPE increases cancer cell sensitivity to chemotherapy and radiotherapy[27] suggesting that CAPE might be combined with current therapy regimens to suppress therapeutic resistant.

CAPE's effect is could be different between solid tumors and hematological malignancies. CAPE was shown to inhibit cell growth of human leukemia cell line HL-60 and reduce Bcl-2, activated caspase 3.[28] Similar results were obtained in studies with U937 leukemia cell line[12] and lymphoblastic B cells PL104 in a dose and time dependent manner.[29] In addition, CAPE inhibited proliferation of ARH-77 multiple myeloma cells and decreased IL-6 secretion.[30]

K562 cells are myeloid and cell line derived from a patient with chronic myeloid leukemia in blast crisis phase. Chromosome number is 1.5 fold more compare to normal cells and cells are resistant to apoptotic mechanisms as they have BCR/ABL fusion gene.[31,32]

In our previous study, we found that CAPE decreases Ara-C resistant in K562 cells.[33] Moreover, we found that CAPE did not change cell cycle in K562 cells (unpublished data) and CAPE suppressed oxidative phosphorylation in K562 cells.[33] These data suggest that CAPE mechanism of action differs in leukemia cells.

AML a malign disease of hematopoietic progenitor cells, is also a heterogenous disease in molecular and phenotypic approach.[34] AML could develop de novo as a result of impaired cell cycle control mechanisms, epigenetic mechanisms that control cell cycle during hematopoiesis and mostly accompanied with chromosomal abnormalities.[35]

Genetic and epigenetic abnormalities are common in AML patients and nearly half of the AML patients have at least one chromosomal aberration.[2] As there are multiple factors involve in AML development, benefit of conventional therapeutics is restricted especially in patients with poor prognosis. Specifically, in elderly patients with poor prognosis and accompanying co-morbid diseases the use of cytotoxic therapy options are limited and therefore there is an increased need for targeted and selective anti-tumoral drugs.[3]

TET2 gene is located on chromosome 4q24.1 and plays role as an epigenetic regulator. TET2 also suggested as a tumor suppressor gene. TET2 mutation was shown to induce proliferation of progenitor cells and resulted extramedullary hematopoiesis.[22] TET2 gene mutations frequently observed in hematological malignancies and has been linked to poor prognosis and advanced progression of the disease. The frequency of TET2 mutations is 11-24% in AML, 11-26% in myelodysplastic syndrome (MDS), 37-44% in MDS/myeloproliferative neoplasms (MPN).[36] In patients with MDS, the presence of TET2 mutation was associated with poor prognosis and suggested as an independent prognostic value.[37] On the other side, in a meta-analysis study, TET2 mutation did not correlate with survival in MDS patients.[38] In an in vitro study, ectopic expression of TET2 in AML patients' clonal stem cells resulted differentiation.[39]

In this study, we found that 5$\mu$M CAPE treatment increased TET2 mRNA expression almost 2.5 fold in K562 cells. With this result, we speculated how CAPE affects TET2 downregulated cells. For this purpose, we aimed to silence TET2 mRNA expression using shRNA-mediated system. However, we managed to reduce TET2 expression to half. Downregulation of TET2 did not change cell proliferation of K562 cells. Using two different shRNA mediated TET2 downregulated cells, we assessed the activity of CAPE on cell proliferation. It was found that TET2 downregulated K562 cells are more resistant to CAPE treatment. This data suggest that CAPE could effect on K562 cells through modifying TET2 expression. Based on our results and literature review following questions arises; how CAPE increases TET2 expression, what are the upstream regulators, whether this alteration in TET2 expression modify DNA methylation upon CAPE treatment and eventually whether CAPE inhibits cell differentiation. Further experiments will answer the questions and shed light on the mechanism of TET2 on cellular response to chemotherapeutics as wells candidate molecules.

## Conclusion

Our results and data on the literature indicate that increased TET2 gene expression inhibits cell proliferation and induce cell differentiation through decreased DNA methylation. Furthermore, it is well known that CAPE does not affect normal cell proliferation suggesting that it is a tumor selective molecule. In conclusion, It can be speculated that CAPE might be combined with current chemotherapeutics to treat leukemia and/or MDS with its effect on TET2 expression. This hypothesis needs to be confirmed with further studies.

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