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A γ-Tocopherol-Rich Mixture of Tocopherols Maintains *Nrf2* Expression in Prostate Tumors of TRAMP Mice via Epigenetic Inhibition of CpG Methylation[1,2]

Ying Huang,[3,5] Tin Oo Khor,[3,5] Limin Shu,[3] Constance Lay-Lay Saw,[3] Tien-Yuan Wu,[3] Nanjoo Suh,[4] Chung S. Yang,[4] and Ah-Ng Tony Kong[3]*

[3]Department of Pharmaceutics, and [4]Susan Lehman Cullman Laboratory for Cancer Research, Department of Chemical Biology, Center for Cancer Prevention Research, Earnest Mario School of Pharmacy, Rutgers, The State University of New Jersey, Piscataway, NJ

Abstract

Nuclear factor-erythroid 2-related factor 2 (Nrf2) plays a pivotal role in maintaining cellular redox homeostasis and eliminating reactive toxic species. *Nrf2* is epigenetically suppressed due to CpG hypermethylation in prostate tumors from the transgenic adenocarcinoma of the mouse prostate (TRAMP) model. We previously showed that dietary feeding of a γ-tocopherol–rich mixture of tocopherols (γ-TmT) suppressed prostate tumorigenesis in TRAMP mice associated with higher Nrf2 protein expression. We hypothesized that γ-TmT may maintain *Nrf2* through epigenetic inhibition of promoter CpG methylation. In this study, 8-wk-old male TRAMP mice were fed 0.1% γ-TmT or a control diet for 16 wk. The methylation in the *Nrf2* promoter was inhibited in the prostate of the γ-TmT group compared with the control group. Protein expressions of DNA methyltransferase (DNMT), including DNMT1, DNMT3A, and DNMT3B, were lower in the prostate of the γ-TmT group than in the controls. TRAMP-C1 cells were treated with 30 μmol/L of γ-TmT or blank medium for 5 d. The methylation in the *Nrf2* promoter was inhibited in the γ-TmT–treated cells compared with the untreated cells at d 5, and mRNA and protein expressions of Nrf2 and NAD(P)H:quinone oxidoreductase 1 were higher. Interestingly, only DNMT3B was inhibited in the γ-TmT–treated cells compared with the untreated cells. In the aggregate, our findings demonstrate that γ-TmT could inhibit CpG methylation in the *Nrf2* promoter in the prostate of TRAMP mice and in TRAMP-C1 cells, which might lead to higher *Nrf2* expression and potentially contribute to the prevention of prostate tumorigenesis in this TRAMP model. J. Nutr. 142: 818–823, 2012.

Introduction

Nrf2 (nuclear factor-erythroid 2-related factor 2) is a transcription factor that plays pivotal role in maintaining cellular redox homeostasis and elimination of carcinogens and reactive intermediates (1,2). Accumulating evidence has demonstrated that *Nrf2*-decient mice are more susceptible to carcinogenic, inflammatory, and oxidative insults (3,4). Furthermore, it has been found that Nrf2 and its downstream target GST (glutathione-*S*-transferase) are suppressed in human and TRAMP (the transgenic adenocarcinoma of the mouse prostate) prostate cancer associated with excessive reactive oxygen species (5). Higher reactive oxygen species levels could cause genetic and epigenetic instability and transduce a variety of signals for tumor cell survival, proliferation, and invasion (5,6). Although the direct relationship between the loss of *Nrf2* and prostate carcinogenesis is yet to be established, maintaining *Nrf2* expression appears to be critical in retaining cellular adaptability to environmental and endogenous stresses and to delay or prevent the development of prostate cancer.

The suppression of *Nrf2* in prostate tumors of TRAMP mice and TRAMP-C1 cells was found to be caused by CpG hypermethylation in the promoter, especially at the first 5 CpG (7). These CpG are hypermethylated in tumorigenic TRAMP-C1 cells but not in nontumorigenic TRAMP-C3 cells (8). Treatment with DNMT (DNA methyltransferase) inhibitor 5-aza-2′-deoxycytidine and HDAC (histone deacetylase) inhibitor trichostatin A could restore *Nrf2* expression in TRAMP-C1 cells (7). However, it may not be feasible to use 5-aza-2′-deoxycytidine as a cancer chemopreventive agent chronically due to its toxicity, and therefore great effort has been made in looking for effective epigenetic interventions through the use of relatively nontoxic natural compounds (9).

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[5] Y.H. and T.O.K. contributed equally to the project.
* To whom correspondence should be addressed. E-mail: KongT@pharmacy.rutgers.edu.

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Vitamin E refers to a group of lipid-soluble compounds consisting of 8 structurally related tocopherols (α-, β-, γ-, and δ-) and tocotrienols (α-, β-, γ-, and δ-). They are well-known natural antioxidants and are abundant in a variety of foods, including vegetable oils, nuts, and whole grains (10). Epidemiological studies revealed that a higher serum γ-tocopherol level is associated with a reduced risk of prostate cancer (11), but large-scale clinical trials with α-tocopherol supplementation demonstrated inconsistent efficacy against prostate cancer (12,13). γ-TmT (γ-tocopherol-rich mixture of tocopherols) is a by-product of the refining of soybean oil and typically contains 57% γ-tocopherol, 24% δ-tocopherol, 13% α-tocopherol, and 1.5% β-tocopherol. γ-TmT has been shown to inhibit carcinogenesis in different types of cancer, including prostate, colon, lung, and mammary (14–17).

We reported that dietary feeding of 0.1% γ-TmT could inhibit prostate tumorigenesis in TRAMP mice along with higher *Nrf2* expression, but the potential mechanisms remain unknown (17). Hence, the present study was undertaken to investigate whether γ-TmT would maintain *Nrf2* expression by inhibiting CpG methylation in TRAMP mice and TRAMP-C1 cells.

Methods and Materials

Mice. Female heterozygous C57BL/TGN TRAMP mice, line PB Tag 8247NG, and male C57BL/6 mice were purchased from Jackson Laboratory. TRAMP females were crossed with C57BL/6 males and the first or second generation of transgenic males was chosen for the study. The genotype of the offspring was determined by a PCR-based method (18). Mice were housed in cages with wood chip bedding in a temperature-controlled room (20–22°C) with a 12-h-light/-dark cycle, with a relative humidity of 45–55% in Rutgers Animal Care Facility. The study was carried out using an IACUC-approved protocol at Rutgers University.

Mouse study design. To test whether higher *Nrf2* expression in TRAMP prostates after γ-TmT treatment was associated with decreased promoter methylation, we repeated the treatment of 0.1% γ-TmT diet in 8-wk-old TRAMP mice for 16 wk, as previously performed (17). Eight-week-old male TRAMP mice were randomly assigned to treatment (n = 7) and control (n = 6) groups. Mice in the treatment group were fed 0.1% mixed tocopherols in an AIN-93M diet (19). γ-TmT was purchased from Cognis and contained 130.0 mg of α-tocopherol, 15.0 mg of β-tocopherol, 243.0 mg of δ-tocopherol, and 568.0 mg of γ-tocopherol/g. At wk 24, mice were killed by CO_2 asphyxiation and the genitourinary apparatus including the prostate, the seminal vesicles, and the bladder were collected, snap-frozen in liquid nitrogen, and stored in −80°C for further analysis.

Archived prostate tissues of TRAMP mice at the age of 12 wk (n = 4), 18 wk (n = 3), and 24 wk (n = 5) and nontransgenic C57BL/6 mice at the age of 12 wk (n = 2) and 24 wk (n = 2) were used for DNA extraction to determine the methylation status of the *Nrf2* promoter at different ages. These tissues were from our unpublished study and were kept in −80°C for <1 y before DNA collection. Mice were fed Purina Mouse Chow 5015.

Cell culture and treatment. TRAMP-C1 cells were cultured in DMEM containing 10% FBS and antibiotics. Cells were grown at 37°C in a humidified 5% CO_2 atmosphere. γ-TmT was dissolved in DMSO to make a stock solution containing 100 mmol/L total tocopherols consisting of 13.0 mmol/L α-tocopherol, 1.5 mmol/L β-tocopherol, 25.0 mmol/L δ-tocopherol, and 60.5 mmol/L γ-tocopherol. TRAMP-C1 cells were treated with 30 μmol/L of γ-TmT in DMEM containing 1% FBS for 5 d and harvested.

Bisulfite sequencing. Genomic DNA was isolated from TRAMP prostate tissues and TRAMP-C1 cells using a QIAamp mini kit (Qiagen). DNA (800 ng) was bisulfite converted using an EZ DNA Methylation-Gold kit (Zymo Research). TA cloning was performed as previously described (7). For each sample, 5–10 clones were chosen for sequencing. Plasmid DNA was sequenced using T7 primer (Genewiz) at the Rutgers Sequencing Core facility. The methylation percentage was calculated as the number of methylated CpG over the total number of CpG examined.

Western-blot analyses. DNMT are key enzymes catalyzing the addition of the methyl group to cytosine and play a critical role in establishing DNA methylation patterns (20). To investigate whether inhibition of methylation in the *Nrf2* promoter was related to down-regulation of any of DNMT, we determined the protein expression of DNMT in the prostate tissues of TRAMP mice and TRAMP-C1 cells. Two prostate specimens in the same group were combined for protein extraction. The detailed procedure of Western blotting was previously described (7). Protein bands were visualized by Supersignal West Femto (Pierce) and documented by Gel Documentation 2000 system (Bio-Rad). Protein expressions were semiquantitated by densitometry using ImageJ program. Antibodies against DNMT1, DNMT3A, and DNMT3B were purchased from Imgenex. Antibodies against Nrf2 (sc-722), NQO1 [NAD(P)H:quinone oxidoreductase 1; sc-16464], and β-actin (sc-1616) were purchased from Santa Cruz Biotechnology.

RNA extraction and RT-PCR. RNA was extracted using a Qiagen RNeasy mini kit and converted to cDNA (TaqMan). Conditions for qPCR were described (18). Relative expression was analyzed by a $^{\Delta\Delta}$Ct method using RQ Manager 1.2 and GAPDH expression was used as internal control. The forward and reverse primers for *Nrf2* amplification were 5′-TCACACGAGATGAGCTTAGGGCAA-3′ and 5′-TACAGTTCTGGGCGGCGACTTTAT-3′. Primers for *Nqo1* were 5′-AAGAGCTTTAGGGTCGTCTTGGCA-3′ and 5′-AGCCTCCTTCATGGCGTAGTTGAA-3′. Primers for GAPDH were 5′-TCAACAGCAACTCCCACTCTTCCA-3′ and 5′-ACCCTGTTGCTGTAGCCGTATTCA-3′.

Statistical analyses. Data are mean ± SEM. Palpable tumor incidence was evaluated using the Fisher exact test. The methylation percentages of the *Nrf2* promoter in the archived prostate samples were compared using 1-way ANOVA followed by Tukey's Studentized range test. For all other determinations, Student's t-test or Welch's t-test was used. SAS, version 9.2, was used for all statistical analyses. All P values correspond to 2-sided hypothesis tests and $P < 0.05$ was regarded as significant.

Results

CpG methylation in the Nrf2 *promoter increases during prostate tumorigenesis in TRAMP mice.* In the present study, we examined the methylation pattern of the first 5 CpG in the prostate of TRAMP and nontransgenic mice at different ages. In the prostate of TRAMP mice, the methylation of these CpG significantly increased from 12 to 24 wk (**Fig. 1***A*,*C*), whereas in the prostate of nontransgenic mice, the methylation remained unchanged (Fig. 1*B*,*C*).

Dietary 0.1% γ-TmT inhibits CpG hypermethylation in the Nrf2 *promoter in the prostate of TRAMP mice.* The palpable tumor incidence (**Table 1**) was significantly lower in the γ-TmT group than in the control group, which is consistent with the previous study (17). The methylation of the first 5 CpG in the *Nrf2* promoter was significantly lower in the γ-TmT group than in the control group (**Fig. 2***A*,*B*). *Nrf2* mRNA expression tended to be higher in the γ-TmT group than in the control group (P = 0.069) (Fig. 2*C*).

γ-TmT reverses CpG hypermethylation in the Nrf2 *promoter in TRAMP-C1 cells.* From the above in vivo study,

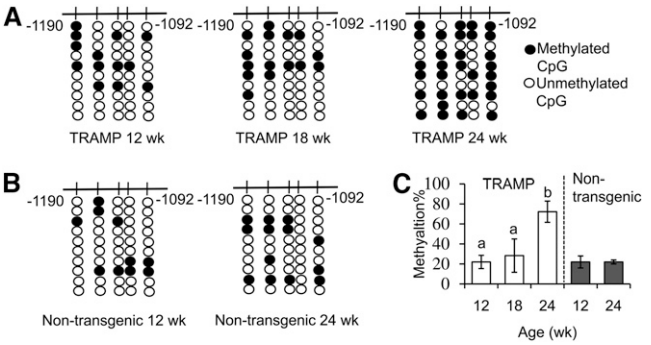

FIGURE 1 Methylation patterns of the first 5 CpG in the *Nrf2* promoter in archived prostate samples from TRAMP (*A*) and nontransgenic (*B*) mice and the methylation percentage (*C*) at various ages. In *C*, data are mean ± SEM, n = 3–5 (TRAMP) or 2 (nontransgenic). In TRAMP mice, means without a common letter differ, $P < 0.05$. TRAMP, transgenic adenocarcinoma of the mouse prostate.

which demonstrated that γ-TmT inhibited CpG methylation in the *Nrf2* promoter, we next investigated whether γ-TmT could reverse CpG hypermethylation in TRAMP-C1 cells. The methylation of the first 5 CpG in the *Nrf2* promoter was inhibited in the γ-TmT–treated cells compared with the untreated cells at d 5 (**Fig. 3**). Cell viability was not affected by the treatment (data not shown).

γ-TmT induces mRNA and protein expressions of Nrf2 *and Nqo1 in TRAMP-C1 cells.* We examined the expression of *Nrf2* to see whether reduced promoter methylation could reactivate gene expression. The mRNA and protein expressions of *Nrf2* and *NQO1* were induced in TRAMP-C1 cells treated with 30 μmol/L of γ-TmT compared with the control cells on d 5 (**Fig. 4**).

γ-TmT suppresses the expression of DNMT in the prostate of TRAMP mice and TRAMP-C1 cells. In the prostate of TRAMP mice, the protein levels of DNMT, including DNMT1, DNMT3A, and DNMT3B, were all lower in the γ-TmT group

TABLE 1 Palpable tumor incidence in TRAMP mice fed control or 0.1% γ-TmT diet for 16 wk[1]

	Current study		Previous study[2]	
Group	*n*	Palpable tumor incidence	*n*	Palpable tumor incidence
Control	6	4/6	17	13/17
γ-TmT	7	0/7*	11	2/11*

[1] *Differs from the control group, $P < 0.05$. TRAMP, transgenic adenocarcinoma of the mouse prostate; γ-TmT, γ-tocopherol-rich mixture of tocopherols.
[2] Adapted from (17).

than in the control group (**Fig. 5***A*). Interestingly, only DNMT3B was suppressed when TRAMP-C1 cells were treated with 30 μmol/L of γ-TmT for 5 d (Fig. 5*B*). These results suggest the potential role of DNMT in CpG methylation and demethylation regulated by γ-TmT.

Discussion

γ-TmT has been shown to inhibit prostate tumorigenesis in TRAMP mice associated with higher *Nrf2* expression (17); however, the molecular mechanism remains unclear. In the present study, we show that γ-TmT treatment prevented CpG hypermethylation in the *Nrf2* promoter in vivo and reversed its hypermethylation in vitro, which might contribute to higher *Nrf2* expression. Tocopherols are extensively studied with respect to their antioxidative, antiinflammatory, and antiproliferative effects (21), yet to the best of our knowledge, their effects on epigenetic modification have not been reported.

The progression of prostate tumorigenesis in the TRAMP model is associated with abnormal DNA methylation events with both locus-specific hypermethylation and global genomic hypomethylation (22). The present study demonstrated that methylation of the first 5 CpG in the *Nrf2* promoter increased during prostate cancer development in TRAMP mice, especially at the late stage (Fig. 1*A*,*C*). These CpG are critical in regulating the expression of *Nrf2*, and the increased methylation may

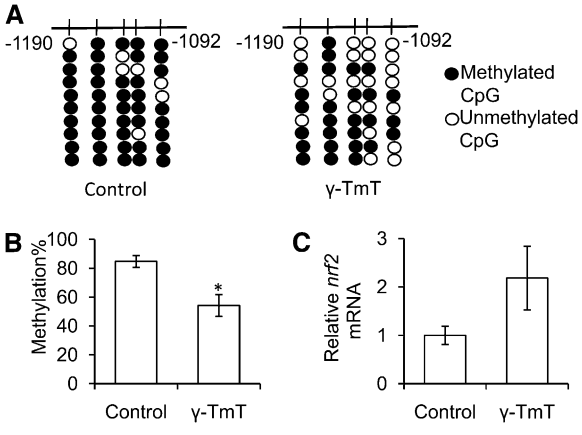

FIGURE 2 Methylation patterns of the first 5 CpG in the *Nrf2* promoter (*A*), the overall methylation percentage (*B*), and the mRNA expression of *Nrf2* (*C*) in the prostate of TRAMP mice fed a control or 0.1% γ-TmT diet. Data are mean ± SEM, n = 4 (control) or 7 (γ-TmT). *Different from control, $P < 0.05$. TRAMP, transgenic adenocarcinoma of the mouse prostate; γ-TmT, γ-tocopherol-rich mixture of tocopherols.

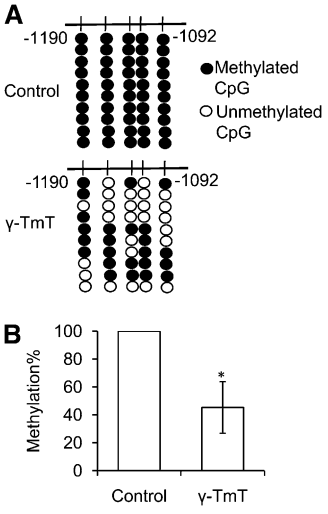

FIGURE 3 The methylation pattern (*A*) and the overall methylation percentage (*B*) of the first 5 CpG in the *Nrf2* promoter in TRAMP-C1 cells following treatment with 30 μmol/L of γ-TmT for 5 d. Data are mean ± SEM, n = 3. *Different from control, $P < 0.05$. TRAMP, transgenic adenocarcinoma of the mouse prostate; γ-TmT, γ-tocopherol-rich mixture of tocopherols.

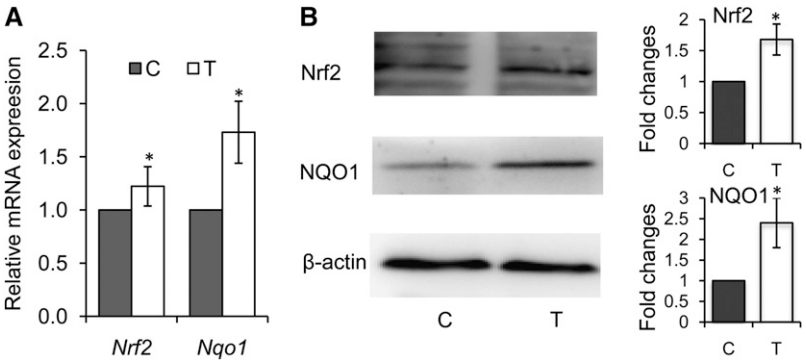

FIGURE 4 The mRNA (*A*) and protein (*B*) expression of *Nrf2* and *NQO1* in TRAMP-C1 cells following the treatment with 30 μmol/L of γ-TmT for 5 d. Three independent experiments were carried out. Data are mean ± SEM, n = 3. *Different from control, $P < 0.05$. C, control; TRAMP, the transgenic adenocarcinoma of mouse prostate; γ-TmT, γ-tocopherol-rich mixture of tocopherols; T, γ-TmT treated.

contribute to the lower *Nrf2* expression in prostate tumors of TRAMP mice, as previously reported (7). *NRF2* is also found to be repressed in human prostate cancer (5); however, future studies are warranted to investigate whether *NRF2* inactivation in human prostate cancer is caused by CpG hypermethylation.

Prostate cancer in TRAMP mice appears to involve excessive oxidative stress, accompanied by increased damage to DNA, protein, and lipid (6). Because Nrf2 plays a central role in adapting the cells to environmental and endogenous stresses (1), the loss of *Nrf2* expression would potentially make the prostate of TRAMP mice more vulnerable to insults, because Nrf2-targeted enzymes such as the SOD, UGT1A1, NQO1, and GST family are also lost during tumorigenesis (5,17,23). γ-TmT inhibited CpG methylation (Figs. 2*A*,*B* and 3) and elevated Nrf2 and its downstream antioxidant enzyme NQO1 in TRAMP-C1 cells (Fig. 4), which could potentially contribute to the prevention against prostate cancer.

Some natural phytochemicals have been shown to reactivate the expression of silenced genes in tumor cells through epigenetic modifications (9,24). Possible mechanisms could be related to inhibition of DNMT and/or HDAC. For instance, green tea polyphenols, sulforaphane, and curcumin have been reported to inhibit both DNMT and HDAC (24–26). In the present study, we showed that dietary γ-TmT feeding suppressed the expressions of all 3 DNMT in TRAMP mice (Fig. 5*A*). Lower expression of DNMT could prevent promoter CpG hypermethylation in the prostate of TRAMP mice, including the *Nrf2* promoter during the early stages of tumorigenesis. Furthermore, metabolites of tocopherols are hypothetical HDAC inhibitors as predicted by molecular modeling (24). We speculate that histone modifications might also contribute to the lower CpG methylation and higher *Nrf2* expression after γ-TmT treatment in vivo. Further study is needed to explore the effect of γ-TmT on HDAC and histone modifications.

The human body preferentially retains α-tocopherol despite the high γ-tocopherol intake from the typical American diet (21). This is achieved in part by the selectivity of the hepatic α-tocopherol transfer protein, which facilitates the entrance of α-tocopherol into the circulatory system, while the non-α-tocopherols undergo fast metabolism mediated by the cytochrome P450 (27). In immunodeficient mice fed 0.1% γ-TmT, α-tocopherol remained the most abundant form in the prostate, though its concentration was not greater than in the control group (28). The concentrations of γ- and δ-tocopherol in the prostate increased by 2- to 3-fold following 0.1% γ-TmT treatment. Another study showed that the urinary excretions of tocopherol metabolites such as γ- and δ-carboxymethyl hydroxychromans dramatically increased in immunodeficient mice following 0.17–0.3% of γ-TmT feeding (29). These tocopherol levels reported in mice suggest that the observed epigenetic effect in the prostate of TRAMP mice in the present study may be attributed to the single or combined effects of γ- and δ-tocopherol and their metabolites.

As a proof-of-concept, we demonstrated that γ-TmT could reverse hypermethylation of the *Nrf2* promoter using TRAMP-C1 cells (Fig. 3). However, DNMT3B, but not DNMT1 or DNMT3A, was suppressed in the γ-TmT–treated cells at d 5 (Fig. 5*B*). There are several possible explanations for the

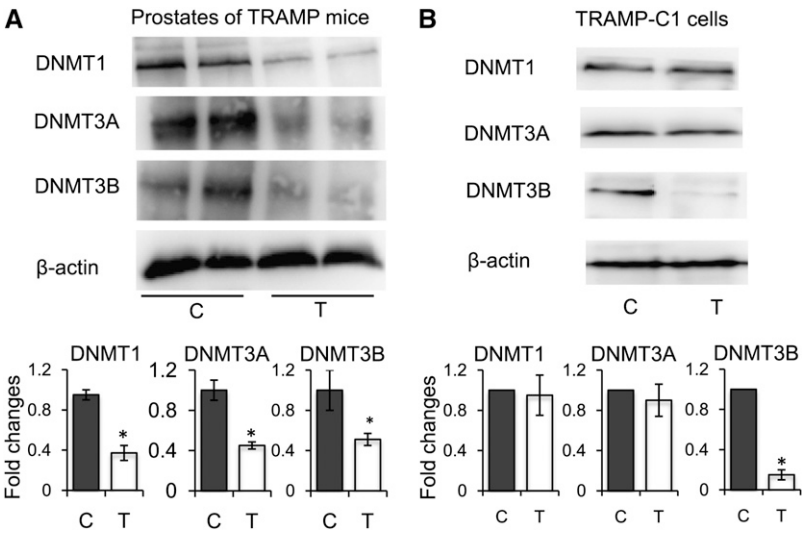

FIGURE 5 Protein expressions of DNMT1, DNMT3A, and DNMT3B in the prostate of TRAMP mice fed a control or 0.1% γ-TmT diet (*A*) and in TRAMP-C1 cells (*B*) treated with 30 μmol/L of γ-TmT for 5 d. Values are mean ± SEM, n = 4–7 (TRAMP mice) or 3 (TRAMP-C1 cells). *Different from control, $P < 0.05$. C, control; γ-TmT, γ-tocopherol-rich mixture of tocopherols; T, γ-TmT treated.

differences between the in vivo and in vitro results. First, the TRAMP study revealed primary prevention, in which γ-TmT blocked the expression of DNMT proteins and the methylation of the *Nrf2* promoter during prostate tumorigenesis. The in vitro study was carried out in a prostate cancer cell line, in which the *Nrf2* promoter is already hypermethylated (7), and the γ-TmT treatment reversed the hypermethylation. Second, prostate tumors from TRAMP mice are a heterogeneous population (8) compared with the TRAMP-C1 cells, which are relatively homogenous and therefore could result in different outcomes upon γ-TmT treatment. Third, the concentrations of different tocopherols and their metabolites in prostate tissues and TRAMP-C1 cells might be different. It has been reported that only a small portion of tocopherols in cell culture medium can be metabolized by human prostate cancer cells (30). Hence, it is likely that TRAMP-C1 cells are mostly exposed to the parent tocopherols as they are supplemented in the medium. In contrast, mice can metabolize tocopherols extensively in vivo, generating high concentrations of carboxychromanol metabolites (29), some of which have been shown to possess superior biological activity compared with the parent tocopherols (31–33).

In summary, in our present study, we showed that γ-TmT prevents CpG methylation in the *Nrf2* promoter in vivo in the prostate of TRAMP mice and reverses hypermethylation of the *Nrf2* promoter in vitro in TRAMP-C1 cells, associated with lower DNMT protein expressions. These epigenetic modifications might contribute to higher *Nrf2* expression, which potentially plays a role in the prevention of prostate tumorigenesis in TRAMP mice.

Acknowledgments

Y.H. discussed, designed, conducted the research, analyzed the data, and wrote the manuscript; T.O.K. discussed, designed, conducted the research, and wrote the manuscript; N.S. and C.S.Y. discussed and designed the research; A.N.K. discussed and designed the research, wrote the manuscript and had primary responsibility for the final content; and L.S., C.L-L.S., and T-Y.W. conducted the research. All authors read and approved the final manuscript.

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