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Pharmacodynamics of curcumin as DNA hypomethylation agent in restoring the expression of Nrf2 via promoter CpGs demethylation

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ABSTRACT

Prostate cancer (PCa) is one of the most deadly malignancies among men in the United States. Although localized prostate cancer can be effectively treated via surgery or radiation, metastatic disease is usually lethal. Recent evidence suggests that the development and progression of human prostate cancer involves complex interplay between epigenetic alterations and genetic defects. We have recently demonstrated that Nrf2, a master regulator of cellular antioxidant defense systems, was epigenetically silenced during the progression of prostate tumorigenesis in TRAMP mice. The aim of this study is to investigate the potential of curcumin (CUR), a dietary compound that we have reported to be able to prevent the development of prostate cancer in TRAMP mice, as a DNA hypomethylation agent. Using bisulfite genomic sequencing (BGS), treatment of TRAMP C1 cells we showed that CUR reversed the methylation status of the first 5 CpGs in the promoter region of the Nrf2 gene. Methylation DNA immunoprecipitation (MeDIP) analysis revealed that CUR significantly reduced the anti-mecyt antibody binding to the first 5 CpGs of the Nrf2 promoter, corroborated the BGS results. Demethylation of Nrf2 was found to be associated with the re-expression of Nrf2 and one of its downstream target gene, NQO-1, one of the major anti-oxidative stress enzymes, both at the mRNA and protein levels. Taken together, our current study suggests that CUR can elicit its prostate cancer chemopreventive effect, potentially at least in part, through epigenetic modification of the Nrf2 gene with its subsequent induction of the Nrf2-mediated anti-oxidative stress cellular defense pathway.

1. Introduction

Unlike genetic alterations, changes in gene expression due to epigenetic regulation can be potentially reversed by chemicals. Gene silencing by promoter hypermethylation has been implicated in the development of many human malignancies including prostate cancer (PCa) [1]. There is increasing evidence that in human PCa, epigenetic alterations occur earlier than genetic defects [2]. Therefore, drugs that target enzymes responsible for DNA methylation and/or histone modification leading to reactivation of epigenetically silenced genes can be useful in cancer prevention and treatment. The histone deacetylases (HDACs) and DNA methyltransferases (DNMTs) inhibitors have been approved for use in hematological malignancies and are currently in different phases of clinical trials (reviewed in [3,4]). However, adverse side effects have hindered the development of these compounds as a cancer chemopreventive/therapeutic agent.

Despite its poor bioavailability, curcumin (CUR) was found to be a very powerful cancer chemopreventive agent using animal models of different cancers (reviewed in [5]). In our laboratory, we have demonstrated that CUR is effective against the growth and progression of prostate tumor in immunodeficient and TRAMP mice [6,7]. In humans, CUR has shown promising results in a phase II trial involving patients with advanced pancreatic cancer [8]. It has been suggested that the pharmacological effect of CUR is achieved through the accumulation of hydrophobic CUR and its metabolites in tissue as a result of long term oral exposure. Accumulating evidence indicates that CUR may exert its chemopreventive/therapeutic effect through epigenetic modification, which is achievable at lower concentrations [9]. Although the underlying mechanisms remain unclear, CUR has been shown to possess inhibitory effects on HDACs, HATs and more recently DNMT activity through different approaches and systems [10–15].

Abbreviations: PCa, prostate cancer; Nrf2, nuclear factor erythroid-2 (NF-E2) related factor-2; CUR, curcumin; BGS, bisulfite genomic sequencing; MeDIP, methylation DNA immunoprecipitation; anti-mecyt, anti-methycytosine; HDAC, histone deacetylases; DNMTs, DNA methyltransferases; GST, glutathione-S-transferases; NQO1, NAD(P)H:quinone oxidoreductase-1; HO-1, heme oxygenase-1; Gpx, glutathione peroxidases; TIS, translation initiation site; 5-Aza, 5-azadeoxycytidine; TSA, Trichostatin A.

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Epidemiological and experimental evidence have linked oxidative stress and chronic inflammation with neoplastic transformation and carcinogenesis ([16–18]). Previous studies from our as well as other laboratories have demonstrated that nuclear factor erythroid-2 (NF-E2) related factor-2 (Nrf2 or NFE2L2) plays critical roles in defense against oxidative stress [19–27]. Through the antioxidant or the electrophile response element (ARE/EpRE; GTGACNNNGC), Nrf2 regulates the induction of anti-oxidative stress proteins such as phase II detoxification enzymes glutathione-S-transferases (GST), NAD(P)H:quinone oxidoreductase-1 (NQO1), and antioxidant proteins heme oxygenase-1 (HO-1), and glutathione peroxidases (Gpx) [28–30]. Nrf2 deficiency has been shown to be closely correlated with increased susceptibility to carcinogen induced tumorigenesis in mice [31–34]. We have reported that Nrf2 and its target gene HO-1 were attenuated in the skin tumors of mice induced by DMBA-TPA [32]. We also found that the development of prostate tumors in TRAMP mice was associated with gradual down-regulation of Nrf2 as well as its downstream target genes such as NQO-1, UGT1A1 and GSTM1 [35]. Likewise, Frolich et al. found that the expression of Nrf2 and GST mu genes was significantly decreased in TRAMP prostate tumor [36]. Most recently we reported that as PCa progresses in TRAMP mice, there was a progressive loss of expression of Nrf2 and its downstream target genes such as NQO-1, UGT, GST and HO-1 2 [7,35]. Treatments with γ-rich tocopherols [35] inhibited TRAMP mice prostate carcinogenesis with concomitant restoration of Nrf2 and its target genes such as UGT, GST, Gpx and HO-1. The aim of our current study is to investigate the potential of CUR to restore the expression of Nrf2 through DNA demethylation.

2. Materials and methods

2.1. Reagents and cell culture

The CUR used in this study (cat# C1386) contains approximately 70% of CUR as determined using HPLC method by Sigma–Aldrich (St. Louis, MO, USA) (the remaining 30% comprises demethoxycurcumin and bidemethoxycurcumin). TRAMP C1 cells (generously provided by Dr. Barbara Foster, Department of Pharmacology and Therapeutics, Roswell Park Cancer Institute, Buffalo, NY) were maintained in DMEM supplemented with 10% heat-inactivated fetal bovine serum at 37 °C in a humidified 5% CO_2 atmosphere as described previously [37]. Cells were seeded in 10 cm plates for 24 h, and then treated with either 0.1% DMSO (control), azadeoxycytidine (5-Aza) (Sigma–Aldrich, St. Louis, MO, USA) and Trichostatin A (TSA) (Sigma–Aldrich, St. Louis, MO, USA) or different concentrations of CUR in 1% FBS containing medium. The medium was changed every 2 days. For the 5-aza and TSA combination treatment, 500 nM TSA was added to the 5-Aza containing medium on day 4 and then incubated for another 20 h. Cells were harvested for protein, DNA or total RNA analyses on day 5.

2.2. Bisulfite genomic sequencing (BGS)

Genomic DNA was isolated from CUR treated or control TRAMP-C1 cells using the DNeasy tissue kit (Qiagen, Valencia, CA). The bisulfite conversion was carried out with 750 ng of genomic DNA using EZ DNA Methylation Gold Kits following the manufacturer's instructions (Zymo Research Corp., Orange, CA). The converted DNA was amplified by PCR using Platinum PCR SuperMix (Invitrogen, Grand Island, NY) with the primers that amplify the first 5 CpGs located between −1086 and −1226 of the murine Nrf2 gene, with the translation initiation site (TIS) defined as position 1 [37]. The PCR products were purified by gel extraction using the Qiaquick™ gel extraction kit (Qiagen, Valencia, CA), then cloned into pCR4 TOPO vector using a TOPO™ TA Cloning kit (Invitrogen, Grand Island, NY). Plasmids DNA from at least 10 colonies per treatment groups from 3 independent experiments were prepared using QIAprep Spin Miniprep Kit (Qiagen, Valencia, CA) and sequenced (DNA Core Facility, Rutgers/UMDNJ, Piscataway, NJ), as we have previously reported [37].

2.3. Methylation DNA immunoprecipitation (MeDIP) analysis

The MeDIP analysis was performed as previously described with some modifications [38,39]. Briefly, 8 μg DNA extracted from control and CUR treated cells was adjusted to 150 μL using TE buffer, followed by sonication on ice using a Bioruptor sonicator (Diagenode Inc., Sparta, NJ) to shear the DNA to an average size of 300–500 base pairs (bp). One-tenth of the fragmented DNAs was kept as inputs and the remaining DNA was denaturated at 95 °C for 10 min, and followed by immunoprecipitation (IP) in 1× IP buffer (10 mM sodium phosphate pH 7.0, 140 mM NaCl, 0.25% Triton X-100) using anti-methylcytosine antibody (anti-mecyt; Anaspec, Fremont, CA) or negative control antibody (anti-cMyc, Santa Cruz, Santa Cruz, CA) for 2 h at 4 °C, respectively. After the incubation, 30 μL magnetic beads (Cell signaling, Boston, MA) were added, and rotated at 4 °C for another 2 h, the pulled-down DNA beads complex were washed four times using ice cold IP buffer and digested with proteinase K at 50 °C overnight, and followed by DNA purification using miniprep kit from Qiagen (Valencia, CA). One microliter of each of the purified enriched or input DNA was used as template for 30 cycles of PCR amplification using primer set covering the DNA sequence from position −1190 to −1092 of the murine Nrf2 gene in which the first 5 CpGs are located as previously described [37]. The PCR products were then analyzed by agarose gel electrophoresis and visualized using ethidium bromide (EB) staining.

2.4. RNA isolation and reverse transcription-PCR

Total RNA was extracted from the treated cells using the Trizol (Invitrogen, Carlsbad, CA). mRNA expression levels of Nrf2 and NQO1 were determined by quantitative real time-polymerase chain reaction (PCR) (ABI7900HT) using delta delta Ct method. First-strand cDNA was synthesized from 1 μg of total RNA using SuperScript III First-Strand Synthesis System for the subsequent Reverse transcription (RT)-PCR (Invitrogen, Grand Island, NY) according to the manufacturer's instructions. The cDNA was used as the template for PCR reactions performed using Power SYBR Green PCR Master Mix (Applied Biosystem, Carlsbads, CA). The PCR primers to specific genes to be amplified have been reported in our previous publication [37].

2.5. Preparation of protein lyses and Western blotting

The treated cells were harvested using radioimmunoprecipitation assay (RIPA) buffer supplemented with protein inhibitor cocktail (Sigma, St. Louis, MO). The protein concentrations of the cleared lysates were determined using the bicinchoninic acid (BCA) method (Pierce, Rockford, IL), and 20 μg of the total protein were resolved by 4–15% SDS-polyacrylamide gel electrophoresis (Bio-rad, Hercules, CA). After electrophoresis, the proteins were electro-transferred to polyvinylidene difluoride (PVDF) membrane (Millipore, Bedford, MA). The PVDF membrane was blocked with 5% BSA in phosphate-buffered saline-0.1% Tween 20 (PBST), and then sequentially incubated with specific primary antibodies and HRP-conjugated secondary antibodies. The blots were visualized by SuperSignal enhanced chemiluminiscence (ECL) detection system and documented using a Gel Documentation 2000 system

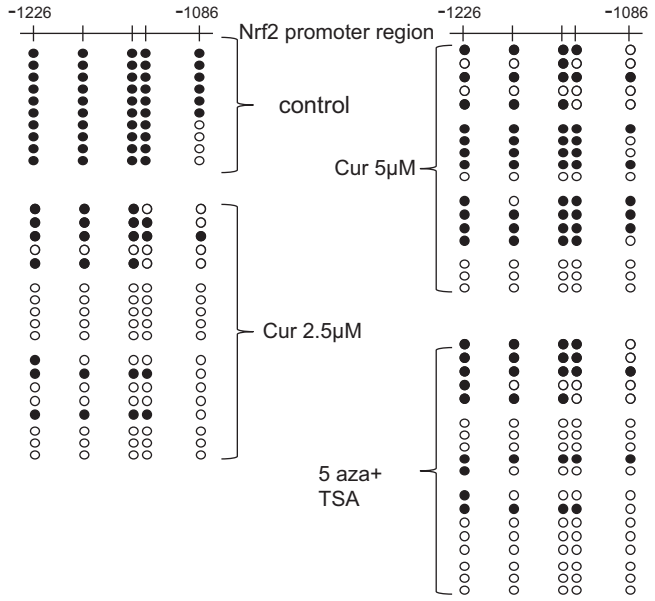

Fig. 1. The methylation patterns and extents of the first 5 CpGs of the promoter of Nrf2 gene were determined using bisulfite genomic sequencing (BGS) as described in Section 2. Black dots indicate methylated CpGs and open circles indicate non-methylated CpGs. 92% of the control TRAMP C1 cells were found to be methylated. Treatment with 2.5 μM, 5 μM or combination of 5-aza/TSA (2.5 and 0.5 μM, respectively) significantly reduces the methylation level to 27, 57.6 and 30%, respectively (Fisher exact test $P < 0.0001$). At least three clones from three independent experiments were selected for BGS.

(Bio-Rad, Hercules, CA). The antibodies, anti-Nrf2, actin and NQO-1 were purchased from Santa Cruz biotechnology (Santa Cruz, CA).

2.6. In vitro methylation assay

The *in vitro* methylation assay was performed as previously described with slight modifications [40]. The substrate DNA for the *in vitro* methylation assay was a 850-bp fragment; −444/+401 relative to the initiation codon from the promoter region of the human $p16^{Ink4a}$ gene. The methylation reaction contained 350–400 ng of the substrate DNA and 4 units of *M.SssI* methylase (0.5 μmol/L, New England Biolabs, Frankfurt, Germany) in a final volume of 50 μL. Inhibitors were added to final concentrations of 5, 50, and 100 μM. Reactions were performed at 37 °C for 1 h followed by 1 h digestion at 60 °C with 30 units of *Bst*UI (New England Biolabs) and analyzed on 2% agarose gels.

3. Results

3.1. Hypermethylation of specific CpG sites in the CpG island of Nrf2 gene in TRAMP C1 cells was reversed by CUR treatment

We have previously reported that the first 5 CpGs in the CpG island of Nrf2 gene are hypermethylated in TRAMP prostate tumors and in the tumorigenic TRAMP C1 cells but not in normal prostate tissues and non-tumorigenic TRAMP C3 cells [37]. We also found that the promoter activity of Nrf2 was significantly suppressed when these 5 CpGs were hypermethylated [37]. To test if CUR treatment can reverse the methylation status of these 5 CpGs on Nrf2 promoter, bisulfite sequencing was performed. In agreement with our previous report, these 5 CpGs was hypermethylated in TRAMP C1 cells (Fig. 1, untreated control, 92% methylation). However, when the cells were treated with either 5 or 10 μM of CUR or a combination of 2.5 μM 5-Aza and 500 nM of TSA for 5 days, methylation of these 5 CpGs was significantly reduced (Fig. 1, 27, 57.6 and 30% methylation, respectively).

MeDIP analysis has been previously shown to be able to enrich methylated DNA in an unbiased manner [38]. Enrichment of methylated DNA fragments can be achieved via immunoprecipitation of DNA obtained from treated and untreated cells with the anti-mecyt antibody that binds specifically to methylated cytosine. The enriched DNA was then purified and used as template to amplify the Nrf2 promoter region that contains the first 5 CpGs. In agreement with the bisulfite sequencing results, MeDIP analysis revealed that CUR significantly reduced the anti-mecyt antibody binding to the first 5 CpGs of Nrf2 promoter (Fig. 2).

3.2. Expression of Nrf2 and its downstream target gene, NQO-1 was induced by CUR

To examine if demethylation of Nrf2 promoter is associated with transcriptional activation of the gene, the mRNA and protein expression levels of Nrf2 were determined. TRAMP C1 cells were treated with 2.5 μM of CUR for 5 days. Using Western blotting and real-time PCR, we found that the expression of Nrf2 was increased in TRAMP C1 cells upon CUR treatment (Fig. 3A and B). 2.5 μM instead of 5 μM of CUR was selected for this experiment since the results from the bisulfite sequencing showed that 2.5 μM of CUR have a better demethylation effect than 5 μM of CUR.

3.3. CUR inhibits the activity of recombinant CpG methylase M.SssI

DNA methylation, which involves the addition of a methyl group at the carbon-5 position of cytosine residues in the DNA, is mediated by a family of enzymes known as DNA methyltrans-

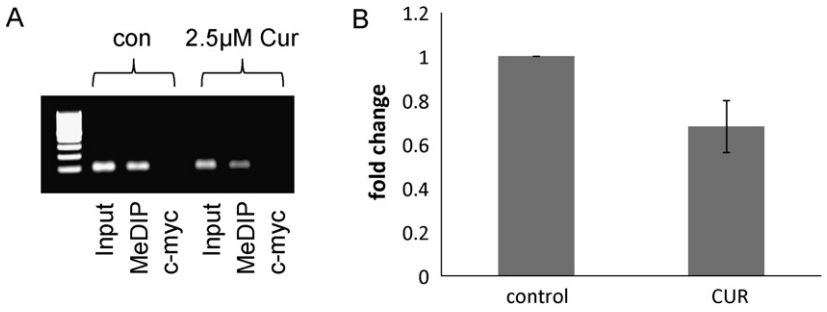

Fig. 2. MeDIP (methylation DNA immunoprecipitation) analysis was performed as described in Section 2. Briefly, 8 μg genomic DNA extracted from control or CUR treated TRAMP C1 cells were sonicated, denatured and subjected to DNA immuniprecipitation (IP) with anti methyl cytosine antibody. (A) Semi-quantative PCR was performed to compare the immunoprecipitated DNA with their inputs and negative control (c-myc as non-specific binding control). Primers covering the first 5 CpGs in Nrf2 promoter region were used; (B) the bands (MeDIP) were visualized using a Gel Documentation 2000 system (Bio-Rad, Hercules, CA) and quantified using Quantity One software. Bars represent mean fold change ± SDEV from 3 independent experiments (normalized with inputs and compared to control value).

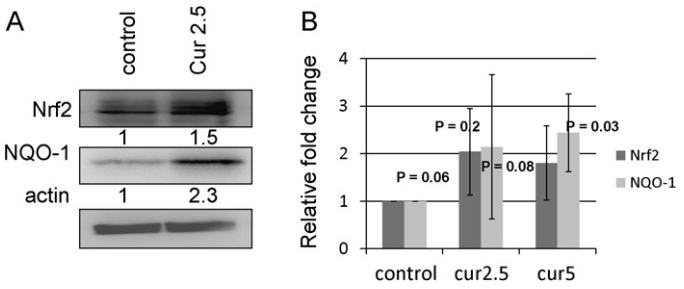

Fig. 3. The mRNA and protein expression level of Nrf2 and NQO-1 determined using real-time PCR and Western blotting. The (A) protein and (B) mRNA expression level of Nrf2 and its target gene NQO-1. The bands were visualized using a Gel Documentation 2000 system (Bio-Rad, Hercules, CA) and quantified using Quantity One software.

ferases (DNMTs) [41]. There are three major DNMTs identified in human including the two *de novo* methyltransferases (DNMT3a and DNMT3b) and the maintenance methyltransferase (DNMT1). To examine if the demethylation effect of CUR could be mediated through transcriptional activation of DNMTs, real-time PCR as well as Western Blotting were performed using total RNA and protein extracted from CUR treated and control TRAMP C1 cells. We found that CUR treatment has no effect on either the mRNA or protein expression level of DNMT1, 3A and 3B (data not shown). To examine if CUR can inhibit the activity of DNA methyltransferase, a cell-free *in vitro* DNA methylation assay was performed. The purified recombinant CpG methylase *M.SssI*, an enzyme shown to possess strong activity and significant structural similarities with the DNMT1 catalytic domain, was used in this assay. As described in Materials and Methods, a 850-bp PCR fragment from the promoter region of the human p16Ink4a gene was used as a substrate for the DNA methyltransferase which was subsequently digested by the methylation-sensitive restriction enzyme BstUI. Our result shows that CUR inhibited DNA methyltransferase activity in a dose-dependent manner as demonstrated by the increase of unmethylated fragment (unprotected, smaller fragments) and decrease of methylated (protected larger) (Fig. 4).

4. Discussion

Accumulating evidence suggests that some dietary chemopreventive phytochemicals may prevent cancer by modifying epigenetic processes in the cells [42–44]. Selenium, a potent cancer chemopreventive agent has been reported to be able to induce global DNA hypomethylation and re-activate epigenetically silenced tumor suppressor genes through inhibition of DNMT activity [45]. Isothiocyanates (ITCs), such as sulforaphane and PEITC from cruciferase vegetables and allyl compounds from garlic, have all been reported as potent HDAC and/or DNMT inhibitors [46–48]. In addition, polyphenols such as (−)-epigallocatechin 3-gallate (EGCG) from green tea and genistein from soybean have also been shown to inhibit DNMTs *in vitro* [43]. Recently, the potential of CUR as an epigenetic modifier has been delineated based on its inhibitory effect on HDACs, HATs and DNMTs [49]. Molecular docking studies showed that the human HDAC8 inhibitory effect of CUR is comparable to Trichostatin A and vorinostat but stronger than valproic acid and sodium butyrate [10]. Likewise, Liu et al. reported that CUR suppressed the expression of HDAC1, 3, and 8 protein levels in a dose-dependent manner associated with increased acetylated histone H4 level [50]. CUR has also been identified as a specific inhibitor of p300/CBP HAT activity [14,51,52]. Using a molecular docking approach, CUR has been shown to exert its DNA methyltransferase I inhibitory effect through covalent binding to the catalytic thiolate of C1226 of DNA methyltransferase I [53]. Subsequent validation experiments demonstrated that CUR has an IC50 of 30 nM in inhibiting *M.Sss1* activity. A recent report from Jha et al. showed that CUR can reverse the hypermethylation leading to activation of the RARβ2 gene in cervical cancer cell lines [54]. However, Liu et al. found that demethoxycurcumin and bisdemethoxycurcumin but not CUR demethylate the WIF-1 promoter region in A549 cells [55]. Given all the promising data indicating the possible epigenetic effects of CUR, hence we proceeded to test the hypothesis whether CUR treatment could reactivate the expression of Nrf2 gene in TRAMP C1 cells through promoter DNA demethylation. Nrf2 was selected as a target because: (1) we as well as others have previously shown that Nrf2 was epigenetically silenced during the development of prostate cancer in TRAMP mice [37]; (2) the first 5 CpGs of Nrf2 promoter region were found to be hypermethylated in prostate tumors from TRAMP mice and TRAMP C1 cells; and (3) the cancer chemopreventive effect of some of the dietary compounds was associated with concomitant restoration of Nrf2 and its target genes such as UGT, GST, Gpx and HO-1 [35].

As one of the most precise methods to identify promoter methylation, bisulfite sequencing was used to dissect the effect of CUR on the methylation status of 5 CpGs of the Nrf2 promoter region. This observation is further supported by the MeDIP analysis that CUR significantly reduces the anti-mecyt antibody binding to the first 5 CpGs of the Nrf2 promoter. Our results show that CUR, at its sub-toxic doses (2.5 and 5 μM) was as effective as 5-AZA/TSA combination in demethylating the first 5 CpGs of the Nrf2 promoter region. Furthermore, the demethylation of these CpGs was associated with increased expression of Nrf2 and its downstream target gene, NQO-1. To investigate the underlying mechanisms by which CUR exerts its DNA demethylation effect, we examined the effect of CUR on the expression of DNMT1, DNMT3a and 3b. However, CUR was found to be possess minimal, if any, effect on the expression of DNMTs both at mRNA and protein levels (data not shown). On the other hand, in agreement with previous finding [53], CUR significantly inhibited the enzymatic activity of CpG methylase *M.SssI* in a dose dependent manner (Fig. 4).

CUR is a multi-targeting agent as evidenced by its ability to interact with at least 33 different proteins covering different signaling pathways [56,57]. CUR is known as a strong activator of Nrf2-mediated transcription of the ARE-luciferase reporter gene as well as an inducer of endogenous Nrf2 protein. For the first time, we showed that CUR can modulate the expression of Nrf2 through epigenetic pathways. Our findings also shed some light on the possible mechanisms by which CUR exert its cancer chemopreventive effect in TRAMP mice [7]. We have previously reported that Nrf2 and its downstream target genes are gradually down-regulated during the prostate tumorigenesis in TRAMP mice [35]. Therefore, the ability of CUR to epigenetically restore the expression of these genes could play an important role in preventing the progression and development of prostate cancer

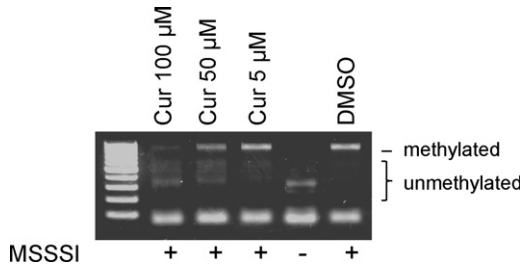

Fig. 4. *In vitro* methylation assay. CUR inhibits *M.SssI* DNA methyltransferase activity in a dose dependent manner as demonstrated by the increase of unmethylated fragment (unprotected, smaller fragments) and decrease of methylated (protected larger).

in TRAMP mice. Based on these data, we draw a possible conclusion that CUR is a potent hypomethylation agent that restores the epigenetically silenced Nrf2 gene, as well as potentially other genes, in TRAMP C1 cells through DNA demethylation. Our findings would provide important information for the future clinical development of CUR as well as other cancer chemopreventive phytochemicals as a cancer epigenetic modifying chemopreventive and therapeutic agents.

Acknowledgements

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