Epigenetic Reactivation of RASSF1A by Phenethyl Isothiocyanate (PEITC) and promotion of apoptosis in LNCaP cells

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Abstract

Epigenetic silencing of tumor suppressor genes is a phenomenon frequently observed in multiple cancers. Ras-association domain family 1 isoform A (*RASSF1A*) is a well-characterized tumor suppressor that belongs to the Ras-association domain family. Several studies have demonstrated that hypermethylation of the *RASSF1A* promoter is frequently observed in lung, prostate, and breast cancers. Phenethyl isothiocyanate (PEITC), a phytochemical abundant in cruciferous vegetables, possesses chemopreventive activities; however, its potential involvement in epigenetic mechanisms remains elusive. The present study aimed to examine the role of PEITC in the epigenetic reactivation of *RASSF1A* and the induction of apoptosis in LNCaP cells. LNCaP cells were treated for 5 days with 0.01% DMSO, 2.5 or 5 μM PETIC or 2.5 μM azadeoxycytidine (5-Aza) with 0.5 μM trichostatin A (TSA). We evaluated the effects of these treatments on CpG demethylation using methylation-specific polymerase chain reaction (MSP) and bisulfite genomic sequencing (BGS). CpG demethylation was significantly enhanced in cells treated with 5 μM PEITC and 5-Aza+TSA; therefore, the latter treatment was used as a positive control in subsequent experiments. The decrease in *RASSF1A* promoter methylation correlated with an increase in expression of the *RASSF1A* gene in a dose-dependent manner. To confirm that promoter demethylation was mediated by DNA methyltransferases (DNMTs), we analyzed the expression levels of DNMTs and histone deacetylases (HDACs) at the gene and protein levels. PEITC reduced DNMT1, 3A and 3B protein levels in a dose-dependent manner, and 5 μM PEITC significantly reduced DNMT3A and 3B protein levels. HDAC1, 2, 4 and 6 protein expression was also inhibited by 5 μM PEITC. The combination of 5-Aza and TSA, a DNMT inhibitor and a HDAC inhibitor, respectively, was used as a positive control as this treatment significantly inhibited both HDACs and DNMTs. The function of *RASSF1A* reactivation in promoting...
apoptosis and inducing G2/M cell cycle arrest was analyzed using flow-cytometry analysis with Annexin V and propidium iodide (PI). Growth inhibition effect on LNCaP cells were investigated by colony formation assay. In addition, we analyzed p21, caspase-3 and 7, Bax, and Cyclin B1 protein levels. Flow-cytometry analysis of cells stained with PI alone demonstrated that 5 μM PEITC promotes early apoptosis and G2/M cell cycle arrest. Flow cytometry analysis of cells stained with Annexin V and PI also demonstrated an increased proportion of cells in early apoptosis in cells treated with 5 μM PEITC or 5-Aza with TSA. PEITC and efficiently inhibit colony numbers and total area. In addition, 5 μM PEITC significantly enhanced p21, caspase-3, 7 and Bax levels and reduced Cyclin B1 expression compared with the control group. Collectively, the results of our study suggest that PEITC induces apoptosis in LNCaP cells potentially by reactivating RASSF1A via epigenetic mechanisms.

Graphical abstract
Schematic illustrating the role of PEITC in epigenetically regulating the transcription of RASSF1A and its role in cell cycle and apoptosis.

Keywords
RASSF1A; Epigenetics; Demethylation; Histone deacetylation; Apoptosis; Phenethyl Isothiocyanate; Tumor suppressor; Prostate cancer

1. Introduction
Prostate cancer (PCa) is one of the most frequent causes of cancer-related deaths in men in the United States. The most recent data from 2015 indicate that greater than 220,800 new cases of PCa are diagnosed and an estimated 27,540 cases of prostate cancer-related deaths occur in men in the US each year[1, 2]. Lifestyle factors, such as diet and nutrition, play a significant role in the pathogenesis of PCa, and multiple studies have revealed that intensive nutrition and lifestyle changes might modulate gene expression in the prostate. These findings highlight the potential role of dietary phytochemicals in the treatment of PCa[3–5]. Studies evaluating epigenetic modifications in cancer, specifically those that lead to the inactivation or silencing of key regulatory genes, such as tumor suppressor genes, has led to the realization that genetic and epigenetic changes play a critical role in tumorigenesis[6]. Recent advances in the field of epigenetics, which are partly due to next generation sequencing techniques, have redirected the focus of cancer researchers. These advances have led to the realization that the development of this deadly disease involves interplay between genetic alterations and epigenetic aberrations. DNA methylation, histone modifications,
nucleosome positioning and non-coding RNAs, such as microRNAs, are specific epigenetic aberrations that induce the expression of oncogenes or silence the expression of tumor suppressor genes[7].

In recent years, epigenetic silencing of tumor suppressor genes has gained much attention in cancer research, and the significance of this phenomenon in driving tumorigenesis is now well established. Recent developments in this field have led to the hypothesis that reactivating tumor suppressor genes that have been silenced by promoter methylation might be an effective targeted therapy for treating cancer [8]. Several studies have demonstrated that the epigenetic silencing of several genes, including RARβ2, RARβ4, RASSF1A, CDH13, APC, CDH1, FHIT, CDKN2A and Nrf2, is due to increased promoter methylation exclusively in prostate tumor tissues[9–13]. Various changes in DNA methylation patterns can be detected before PCa becomes invasive, suggesting that epigenetic changes are pivotal events in tumor progression and that epigenomic changes play a critical role in PCa[14].

The RASSF1A gene, a well-characterized tumor suppressor, belongs to the Ras-association domain family of genes and is located on chromosome at 3p21.3. The RASSF1 gene encodes several isoforms, including RASSF1A, RASSF1B, and RASSF1C, all of which are generated from alternative mRNA splicing and promoter usage[15]. RASSF1A transcriptional silencing is frequently observed feature in multiple types of cancer as a result of promoter hypermethylation; however, few reports are available describing the mechanisms underlying this phenomenon[16–19]. RASSF1A transcription is suppressed by hypermethylation in breast, ovarian, gastric, nasopharyngeal, and bladder cancers as well as neuroblastoma and renal cell lines[20–25]. Hypermethylation of the RASSF1A gene at CpG islands has been observed in 70% of prostate cancer cases, and the restoration of RASSF1A expression inhibited tumorigenesis in prostate and renal tumor cell lines [26, 27]. The tumor suppressor function of RASSF1A has been confirmed by studies demonstrating that the exogenous expression RASSF1A decreased colony formation, suppressed anchorage-independent growth and reduced tumor formation in lung, kidney and breast cancers in vivo. In light of the tumor suppression functions of RASSF1A and the observation that restoring RASSF1A expression in tumor cell lines decreases tumorigenicity, restoring RASSF1A expression and elucidating the mechanism by which hypomethylation occurs have significant implications in the context of preventing tumor growth[22, 26, 28]. Recent meta-analyses have revealed that RASSF1A methylation in tumor tissues was directly correlated with the Gleason Score (GS), serum prostate-specific antigen (PSA) level and tumor stage in PCa, indicating that RASSF1A promoter methylation might be a potential biomarker for prostate cancer prognosis and therapy[29, 30]. Several studies have described the effect of phytochemicals, such as curcumin and mahanie, in reversing RASSF1A promoter hypermethylation and restoring RASSF1A expression in breast cancer and prostate cancer cells[31, 32].

Phenethyl isothiocyanate (PEITC), an abundant component of cruciferous vegetables, such as broccoli and watercress, exerts chemoprevention activity by modulating epigenetic modifications[33, 34]. PEITC is a novel epigenetic regulator of both histone deacetylases (HDACs) and DNA methyltransferases (DNMTs), factors that govern the expression and suppression of several genes, including GSTP1 and p21 [35–37]. Recent studies
demonstrated that PEITC is an effective HDAC inhibitor and reduces HDAC enzyme activity. These events induce growth arrest and apoptosis in cancer cells both in vitro and in vivo [35, 38]. A recent study revealed that PEITC regulated H3-acetylation (H3-Ac) and site-specific H3 lysine methylations (H3K27me3 and H3K9me2), which are epigenetic modifications associated with attenuating cell proliferation in a concentration- and time-dependent manner in human colon cancer cells [39].

We hypothesized that PEITC induces RASSF1A promoter demethylation in LNCaP cells and that epigenetic reactivation of RASSF1A induces apoptosis in LNCaP cells. To test this hypothesis, we treated LNCaP cells with 0.01% DMSO, 2.5 and 5 μM PEITC or 2.5 μM azadeoxycytidine (5-Aza) with 0.5 μM trichostatin A (TSA). The demethylating effect of the test compounds was assessed using the methylation-specific polymerase chain reaction (MSP) and bisulfite genomic sequencing (BGS). We also evaluated the reactivation of RASSF1A expression and the role of RASSF1A activation in promoting apoptosis. The results of our BGS and protein expression analyses indicate that PEITC reactivates RASSF1A expression by promoting demethylation and inhibiting HDAC activation.

2. Materials and methods

2.1. Cell culture, reagents and cell viability assay

The human prostate cancer cell line LNCaP was obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). LNCaP cells were maintained in RPMI-1640 medium (Gibco) supplemented with 10% fetal bovine serum (FBS) (Gibco, Grand Island, NY). The cells were grown at 37 °C in a humidified atmosphere with 5% CO₂. PEITC, 5-Aza, TSA, bacteriological agar, puromycin, and ethidium bromide were purchased from Sigma–Aldrich (St. Louis, MO, USA). The Annexin V and propidium iodide (PI) apoptosis kit was purchased from Life Technologies. Then, 3.5 × 10⁴ LNCaP cells/ml were seeded in 10-cm tissue culture plates for 24 h and treated with DMSO 0.01% (negative control), PEITC (2.5 μM or 5 μM) or 5-Aza with TSA (2.5 μM and 0.5 μM, respectively) (positive control) for 5 days. The drugs and media were replenished every 2 days. TSA (0.5 μM) was added to the 5-Aza treatment group 20 h prior to harvesting the cells.

LNCaP cells were seeded in 96-well plates at a density of 1,000 cells per well. After 24 h, the cells were then treated with PEITC (1–10μM) for 1 and 5 days. The medium was changed every other day for the 5 day group. MTS assay was performed using the Cell Titer 96 Aqueous One Solution Cell Proliferation Assay Kit as described previously (results shown in supporting information).

2.2. DNA methylation analysis

LNCaP cells were plated in 10-cm plates for 24 h and subsequently treated with 0.1% DMSO (control), 2.5 μM 5-Aza and 500 nM TSA, or 2.5 or 5 μM PEITC for 5 days. The medium was changed every other day. For cells treated with 5-Aza and TSA, 500 nM TSA was added 20 h before the cells were harvested. On day 5, the cells were harvested for further analyses. Genomic DNA was isolated from the treated cells using the QIAamp DNA Mini Kit (Qiagen, Valencia, CA, USA). Next, 600 ng of genomic DNA was used as a
template for bisulfite conversion using the EZ DNA Methylation Gold Kit (Zymo Research Corp., Orange, CA, USA) following the manufacturer's instructions. To obtain products for sequencing, the converted DNA was amplified using semi-nested PCR as previously described by Dammann et al. [18]. Briefly, the DNA sequences were amplified by mixing bisulfite-converted DNA (500 ng) with primer MU379 (50 pmol) and primer ML730 (50 pmol) in reaction buffer (20 μl) containing dNTPs (200 μM each dNTP) using Platinum PCR Taq DNA polymerase (Invitrogen, Carlsbad, CA, USA). The reaction conditions consisted of 30 cycles of 95 °C for 1 min, 55 °C for 1 min and 74 °C for 2 min. A semi-nested PCR using the amplified products at a 1:50 dilution, the internal primer ML561 and primer MU379 was performed using similar PCR conditions as described above. The PCR products containing bisulfite-resistant cytosines were ligated into the pCR2.1 vector (Invitrogen), and several clones were sequenced to confirm the presence of the amplified DNA. A minimum of 10 clones per biological replicate from each treatment group were randomly selected and sequenced (Genewiz, Piscataway, NJ, USA). The percentage of methylated CpG sites was calculated by dividing the number of methylated CpG sites by the total number of CpG sites in the DNA sequence. The primer sequences used for BGS are listed in Table 1.

Methylation-specific PCR (MSP) was performed using the bisulfite-converted genomic DNA as template. The primer sequences used for the PCR amplification of the methylated (M) and unmethylated regions of the RASSF1A gene are listed in Table 1. The amplification products were separated on a 2% agarose gel by electrophoresis and visualized using ethidium bromide staining and a Gel Documentation 2000 system (Bio-Rad, Hercules, CA, USA). The densities of the bands were quantified using ImageJ software (Version 1.48d; NIH, Bethesda, Maryland, USA).

2.3. RNA isolation and qPCR

Total RNA from the treatment and control groups was extracted using the RNeasy Mini Kit (Carlsbad, CA), and the mRNA was quantified using a NanoDrop 2000. Approximately 600 ng of mRNA was reverse-transcribed into cDNA using Taqman RT reagents. The relative expression levels of RASSF1A, DNMT1, DNMT3A and DNMT3B mRNA were determined using qPCR with the cDNA template and the Power SYBR Green PCR Master Mix (Applied Biosystems, Carlsbad, CA, USA) in an ABI7900HT system (Applied Biosystems). The sequences of the forward and reverse primers used to analyze these genes are listed in Table 1.

2.4. Protein lysate preparation and Western blot assays

Protein lysates were prepared using radio immunoprecipitation assay (RIPA) buffer (Sigma–Aldrich, St. Louis, MO, USA) supplemented with a protein inhibitor cocktail (Sigma–Aldrich). Total protein was quantified using the bicinchoninic acid (BCA) method (Pierce, Rockford, IL, USA). Briefly, 20 μg of total protein were separated using 4 to 15% sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (Bio-Rad, Hercules, CA, USA) and electro-transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, MA, USA). After blocking with 5% BSA (bovine serum albumin; Fisher Scientific, Pittsburgh, PA, USA) in Tris-buffered saline with 0.1% Tween 20 (TBST)
(Boston Bioproducts, Ashland, MA, USA), the membranes were sequentially incubated with specific primary antibodies and subsequently incubated with horseradish peroxidase-conjugated secondary antibodies. The blots were visualized using the Supersignal West Femto Chemiluminescent Substrate (Pierce, Rockford, IL, USA) and visualized using a Gel Documentation 2000 system (Bio-Rad, Hercules, CA, USA). Densitometry of the bands was analyzed using ImageJ (Version 1.48d; NIH). The primary antibodies were obtained from the following sources: anti-β-ACTIN from Santa Cruz Biotechnology (Santa Cruz, CA, USA); anti-DNMT 3A and 3B from IMGENEX (San Diego, CA, USA); and anti-HDAC1, 2, 4, 6, anti-Cyclin B1 and anti-Bax from Cell Signaling Technology (Boston, MA, USA). The secondary antibodies were purchased from Santa Cruz Biotechnology.

2.5. Flow cytometry analysis of cell cycle distribution and apoptosis

PEITC- or 5-Aza-treated LNCaP cells were stained with PI (Sigma–Aldrich Co., St. Louis, MO) or PI and Annexin V-FITC (Life Technologies) and analyzed by flow cytometry using a Beckman Coulter Gallios Flow Cytometer (Brea, CA) in The Flow Cytometry/Cell Sorting & Confocal Microscopy Core Facility at Rutgers University. Briefly, LNCaP cells (3.5 × 10⁴ cells/ml) were seeded in 10-cm plates, allowed to attach overnight and treated (as previously described) with the indicated drugs. Then, the cells were trypsinized, and the pellet was collected by centrifugation at 700 ×g for 5 min. The cells were fixed with 3 ml of ice-cold 70% ethanol for 48 h at 4 °C prior to the cell cycle analysis. The cells were rinsed twice with phosphate-buffered saline (PBS), treated with RNase A (1 mg/ml) for 30 min at 37 °C, stained with 5 μl of 1 mg/ml PI in the dark at room temperature for 30 min and analyzed according to the manufacturer’s instructions. Following the incubation, the samples, including floating and adherent cells, were collected and prepared for the analysis of apoptosis using the Annexin V-FITC/PI apoptosis detection kit according to the manufacturer’s instructions.

2.6 LNCaP Colony Formation Assay

LNCaP cells were seeded at 800 cells/well density in 6 well dishes. Cells were treated with PEITC (2.5μM, 5μM, 10μM) or 0.1% DMSO (control) every 3 days after seeding. Two weeks later, medium were removed from each of the dishes by aspiration. Cells in each well were washed with 2 mL PBS and fixed by 0.5 mL methanol for 30 minutes. Methanol was removed and the cells were washed by PBS. The cells were stained with 5 mL 0.01% (w/v) crystal violet in dH₂O for 30 minutes. Excess crystal violet was washed out with dH₂O and kept dry. Digital images of the colonies were obtained using a Nikon Eclipse E600 microscope (Micron-optics, NJ). The number and total area of colonies containing more than 50 individual cells were counted by ImageJ (Version 1.48d; NIH, Bethesda, Maryland, USA).

2.7. Statistical analysis

The data are presented as the mean ± standard error of the mean (SEM). Statistical analyses were performed using Student’s t-test and one way ANOVA using Info Stat statistical software program. P-values less than 0.05 were considered statistically significant and are indicated with *. P-values less than 0.01 are indicated with #.
3. Results

3.1 PEITC induced RASSF1A expression in LNCaP cells by inducing promoter demethylation

Hypermethylation of the 16 CpG sites in the RASSF1A promoter region has previously been observed in multiple prostate cancer cell lines, including LNCaP cells[40]. The reversal of RASSF1A promoter hypermethylation in LNCaP cells treated with PEITC was analyzed using MSP and BGS via the semi-nested PCR method. The extent of methylation in MSP was quantified by analyzing the density of the gel bands. The results of the MSP analysis revealed high levels methylated DNA in untreated LNCaP cells, suggesting that the RASSF1A promoter was hypermethylated in untreated cells. However, in the cells treated with PEITC (2.5 or 5 μM) or 5-Aza (2.5 μM) and TSA (0.5 μM), an increase in unmethylated DNA regions was noted compared with the control (Figure 1A). To further analyze the extent of promoter methylation, we used BGS to analyze the 16 CpG sites (Figure 1B) in fragment containing the 3Sp1 consensus binding sites and the putative transcription and translation initiation sites present in Exon 1α (−197 and +438). The Genbank accession number of the cDNA sequence is AF132675 [18, 41]. In the control group, an average 98.1% of the 16 CpG sites were methylated. However, in cells treated with 2.5 μM or 5 μM PEITC, the percentage of methylated CpG sites decreased to 93.3% and 90.7%, respectively, indicating that CpG demethylation was induced in cells treated with PEITC compared with the control group. The percent of methylated CpG sites in the positive control group treated with 5-Aza (2.5 μM) and TSA (0.5 μM) was 83.6% (Figure 1C). The data obtained from MSP and BGS demonstrated that 5 μM PEITC significantly reduced CpG methylation in the RASSF1A gene. Previous studies have reported that reactivation of RASSF1A expression is mediated by promoter demethylation in various types of cancers [31, 42, 43]. We hypothesized that PEITC-induced RASSF1A promoter demethylation increases RASSF1A expression in LNCaP cells. Quantitative polymerase chain reaction (qPCR) analysis revealed that RASSF1A expression was significantly enhanced following treatment with 2.5 μM and 5 μM PEITC in a dose-dependent manner, suggesting that the reactivation of RASSF1A resulted from promoter demethylation (Figure 1D). Treatment with 5-Aza (2.5 μM) and TSA (0.5 μM) significantly enhanced RASSF1A expression, consistent with the observation that the RASSF1A promoter was demethylated in these cells (Figure 1D).

3.2 Regulation of DNA methyltransferases by PEITC

To further investigate PEITC-induced demethylation of the RASSF1A promoter, we evaluated the effect of PEITC on DNMTs, which are enzymes that catalyze the addition of methyl group. Given that we observed a decrease in RASSF1A promoter methylation in cells treated with PEITC, we hypothesized that PEITC and 5-Aza might repress the transcription of DNMTs. The results from the qPCR analysis demonstrated that the expression of DNMT1 (Figure 2A) and DNMT3A mRNA was significantly reduced in cells treated with PEITC (5 μM) or 5-Aza (2.5 μM) and TSA (0.5 μM) (Figure 2B). In addition, the expression of DNMT 3B mRNA transcripts was significantly reduced in cells treated with 5 μM PEITC (Figure 2C). However, the levels of all the DNMTs probed were not significantly affected in cells treated with 2.5 μM PEITC. To determine whether the

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reduction in gene expression translated to a reduction in protein expression, we evaluated
DNMT protein levels using Western blot analysis. Consistent with the reduction in gene
expression, the protein levels of all DNMTs evaluated were significantly reduced in cells
treated with 2.5 μM or 5 μM PEITC compared with the control cells. In addition, analysis of
densitometry demonstrated that DNMT protein levels were reduced in the positive control
group of cells treated with 5-Aza as expected (Figure 2D and 2E).

3.3 Regulation of HDAC protein expression by PEITC

HDACs are enzymes that catalyze the removal of acetyl groups, an epigenetic modification
associated with the induction of gene expression. PEITC is an HDAC inhibitor[37] that
promotes apoptosis. We hypothesized that PEITC treatment would alter HDAC expression,
and we used western blot analysis to investigate this hypothesis. Treatment with 5 μM
PEITC or 2.5 μM 5-Aza and 0.5 μM TSA significantly reduced HDAC1, HDAC2, HDAC4
and HDAC6 protein levels (Figure 3A and Figure 3B). PEITC at a concentration of 2.5 μM
also significantly reduced HDAC 4 and HDAC 6 protein levels (Figure 3A and 3B); however
no significant differences in the other HDACs evaluated were noted between the control and
the treatment groups (data not shown). These data indicated that the repression of DNMTs
and HDAC1, 2, 4 and 6 induced by treatment with PEITC in LNCaP cells led to RASSF1A
promoter demethylation and subsequent activation of RASSF1A.

3.4 PEITC-induced RASSF1A expression induces p21, caspase-3, 7 and Bax expression,
reduces Cyclin B1 expression and promotes apoptosis

Based on the results of the western blot analysis, we further hypothesized that the increase in
RASSF1A expression induced by promoter demethylation promotes cell cycle arrest by
increasing expression of the p21 gene (Figure 4A) and promotes apoptosis by increasing
caspase-3, Bax and caspase-7 expression and reducing Cyclin B1 expression (Figure 4B). To
test this hypothesis, we performed flow cytometry analysis to determine the cell cycle
distribution of PI-stained cells. PEITC significantly enhanced the proportion of cells in the
sub-G0 phase, indicating that these cells were undergoing early apoptosis. In addition, a
significant increase in the proportion of cells in the G2/M phase was noted in the PEITC (5
μM) and 5-AZA (2.5 μM) and TSA (0.5 μM) treatment groups, indicating that these cells
were arrested in the G2/M phase of the cell cycle (Figure 4D). To confirm whether the
growth inhibition induced by PEITC resulted from apoptosis, we performed flow-cytometry
analysis of LNCaP cells stained with Annexin V and PI that were treated with PEITC (2.5
μM and 5 μM) and 5-AZA (2.5 μM) for 5 days. The results of this analysis demonstrated
that treatment with PEITC (5 μM), 5-AZA (2.5 μM) and TSA (0.5 μM) increased the
number of cells in early apoptosis (Annexin V – positive cells) from 4.6% in control cells to
11.1% in cells treated with PEITC (5 μM) and 53.7% in cells treated with 5AZA (2.5 μM)
and TSA (0.5 μM) (Figure 4E). No difference was observed in the cells treated with 2.5 μM
PEITC compared with the control group (data not shown). With respect to late apoptosis (PI-
positive cells), a similar trend was observed in cells treated with 5 μM PEITC, which
exhibited a 2-fold increase in the proportion of cells in late apoptosis compared with the
control group (Figure 4E). Together, these results demonstrate that the reactivation of
RASSF1A gene expression by PEITC-induced promoter demethylation promoted cell cycle
arrest and apoptosis.
3.5 PEITC-inhibited colony formation in LNCaP Cells

As colony formation ability of prostate cancer cells was correlated with its aggressive potential[44], the colony formation assay was used to analyze the inhibition effects of PEITC on the growth of LNCaP cells. LNCaP cells without treatment produced 263±53 colonies, upon treating with PEITC 2.5μM, 5μM and 10μM for 2 weeks, the generated colonies were 159±42, 63±4 and 45±24 respectively, which is significantly reduced from the control group (Figures 5A and 5B). The total colony area were also decreased significantly, after treating with PEITC 2.5μM, 5μM and 10μM for 2 weeks, their relative colony areas were 21.6%, 4.3% and 1.5% respectively (Figure 5C). Taken together, PEITC can effectively inhibit colony formation of LNCaP cells.

4. Discussion

RASSF1A is one of the most frequently hypermethylated genes in human cancers, and RASSF1A hypermethylation is associated with the loss of RASSF1A expression in several types of cancer, including lung, breast, bladder and gastric cancer [21, 22, 24, 45, 46]. In contrast to the RASSF1C isoform, RASSF1A is expressed at higher levels in normal tissues than in lung and breast cancer tumor tissues, suggesting the RASSF1A acts as a tumor suppressor[47]. Previous studies reported that the silencing of RASSF1A expression was a frequently observed phenomenon in advanced stages of prostate cancer[27, 30] and that reversing RASSF1A methylation using phytochemicals, such as mahanine and soy phytoestrogens, reactivated RASSF1A transcription[48, 49]. Several studies that evaluated the mechanism underlying RASSF1A inactivation by analyzing CpG methylation and histone acetylation revealed that reduced histone acetylation or H3K4me2 methylation and increased dimethyl-H3-K9 methylation played a crucial role in RASSF1A promoter methylation and the corresponding inactivation of RASSF1A in prostate cancer [50]. Other studies that investigated the tumor suppressor function of RASSF1A by analyzing microtubule dynamics and apoptosis induction revealed that RASSF1A exerted its tumor suppressor effects primarily by restricting cell cycle progression [51]. The tumor-suppressive function of RASSF1A is through the induction of acetylated α-tubulin and modulation of cell migration [52]. Our study showed that PEITC induced promoter demethylation and induced G2/M cell cycle arrest and apoptosis. It is known that proteins are one of the targets for isothiocyanates. Studies have shown that PEITC covalently binds to tubulin and modifies the cysteine residue forming adducts leading to growth inhibition [53]. It is also shown that increase in RASSF1A expression by 5Aza correlated with an increase in acetylated tubulin [54].

The use of PEITC as a chemopreventive agent has been well studied in prostate cancer and colon cancer by our group and several others [55–57]. Recent investigations into the use of PEITC as a demethylating agent and HDAC inhibitor have provided new insights into its role in regulating epigenetic modifications and regulating genes like p21, GSTP1[36, 37]. PEITC used in combination with conventional chemotherapeutics like Paclitaxel and Doxorubicin has shown synergistic effects [38]. Studies have shown that efficacy of Doxorubicin is known to be inhibited by HER2 over expression. However, when treated in combination with PEITC the cell survival was reduced by 50% [58]. Studies have linked...
HER2 overexpression as well as the overexpression of other epidermal growth factors to hypermethylation of RASSF1A [59, 60]. Our hypothesis of RASSF1A demethylation by PEITC could be one of the possible mechanisms by which PEITC enhances the efficacy of doxorubicin. We hypothesized that PEITC induces demethylation of the RASSF1A promoter in LNCaP cells and that reactivating RASSF1A by reversing this epigenetic modification induces apoptosis in LNCaP cells in vitro. Using MSP and BGS analysis, we demonstrated that PEITC promotes the demethylation of the RASSF1A promoter (Figure 1A and 1C). RASSF1A promoter demethylation was also observed in cells treated with 5-Aza and TSA. As treatment with 5-Aza and TSA is known to reverse the methylation of several genes, these agents were used as a positive control [61]. The demethylation of the RASSF1A promoter was partly due to the inhibition of DNMT1, 3A and 3B gene expression that resulted from treatment with PEITC at both concentrations evaluated (2.5 and 5 μM) (Figure 2A, B and C). However, the protein levels of DNMT3A and 3B but not DNMT1 were significantly inhibited by 5 μM PEITC (Figure 2D and 2E). The tumor suppressor function of RASSF1A is attributed to its ability to modulate cell cycle progression and mitotic spindle dynamics [52, 62]. In this study, we observed that PEITC (5 μM) significantly up-regulated RASSF1A gene expression and p21 expression (Figures 1D and 4A), thus resulting in the induction of G2/M cell cycle arrest (Figure 4D). The ability of RASSF1A to induce p21 expression and promote cell cycle arrest at the G1 and G2/M cell cycle phases was previously demonstrated in tumors from immunodeficient mice [62, 63]. Studies have also revealed that the overexpression of RASSF1A caused G2/M arrest and caused localization of microtubules and caused mitotic arrest [64].

Several studies demonstrated that the RASSF1 gene exhibits pro-apoptotic activity and that the RASSF1A isoform exhibits stronger pro-apoptotic activity than the RASSF1C isoform. RASSF1A increases caspase-3/7 activation to promote apoptosis in breast, lung and ovarian tumors and simultaneously down-regulates the expression of cyclin D1[47, 65]. RASSF1A was also found to activate the key apoptosis gene Bax[66, 67]. Our study revealed that promoter demethylation activated RASSF1A gene expression in LNCaP cells treated with 5 μM PEITC. This effect might account for the increase in apoptosis we observed as caspase-3, caspase 7 and Bax protein expression were significantly enhanced and Cyclin B1 decreased in these cells (Figure 4B). Cyclin B1 is a key regulatory protein for transition the cells from G2 to M phase, however overexpressed Cyclin B1 can lead to uncontrolled cell growth and is highly related to carcinogenesis of many cancers such as breast cancer[68], pancreatic cancer[69], colorectal cancer[70], bladder cancer[71]. It is found that reduction of Cyclin B1 can cause cancer cell apoptosis by arresting the cells in G2 phase[72]. Besides increasing p21 expression, PEITC (5 μM) were also able to reduce Cyclin B1 expression (Figure 4B), which further demonstrate the mechanism of G2/M arrest and apoptosis by PEITC.

We further analyzed the pro-apoptotic role of PEITC in LNCaP cells using flow cytometry analysis of Annexin V- and PI-stained normal, necrotic and apoptotic cells and observed a 2-fold increase in apoptotic cells following PEITC treatment (5 μM). This effect was stronger than that observed in the positive control group of cells treated with 5-Aza and TSA, which exhibited a 1-fold increase in the number of apoptotic cells (Figure 4E). To investigate the direct inhibition effect of PEITC on human prostate cancer LNCaP cells, we analyzed the
colony formation influence by PEITC (2.5μM, 5μM and 10μM) (Figure 5A). It is found that after treatment with all the 3 concentrations of PEITC for 2 weeks, the colony numbers and total colony area were decreased dramatically comparing with control group (Figures 5B and 5C). Hence it suggests PEITC treatment can efficiently inhibit LNCaP cells growth and aggression.

This study is one of the first to demonstrate the role of PEITC in activating RASSF1A and promoting apoptosis in vitro. In conclusion, our findings provide new insight into the role of phytochemicals as epigenetic modulators. Here, 5 μM PEITC significantly induced promoter demethylation and consequently increased RASSF1A gene expression. In addition, PEITC-induced activation of RASSF1A promoted apoptosis and cell cycle arrest. These results further emphasize the DNMT and HDAC inhibitory function of PEITC and its role in promoting apoptosis.

5. Conclusion

Our study revealed that PEITC is a potent regulator of DNA methylation that reverses transcriptionally silenced RASSF1A by inducing promoter demethylation via inhibiting DNMTs and HDACs. RASSF1A activation by PEITC in LNCaP cells induced G2/M cell cycle arrest and apoptosis, indicating that RASSF1A is a potential molecular marker that can be targeted by phytochemicals to prevent prostate cancer.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

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<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>PEITC</td>
<td>Phenethyl isothiocyanate</td>
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<tr>
<td>RASSF1A</td>
<td>RAS superfamily protein isoform 1A</td>
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<td>DNMT</td>
<td>DNA methyl transferases</td>
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<td>HDAC</td>
<td>Histone deacetylases</td>
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<td>MSP</td>
<td>methylation specific</td>
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<td>PCR, BGS</td>
<td>bisulfite genomic sequencing</td>
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<td>PCa</td>
<td>Prostate cancer</td>
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References

Uncategorized References


Effect of PEITC on RASSF1A promoter demethylation. LNCaP cells (3.5 x 10^4 cells/ml) were seeded in 10-cm tissue culture plates. LNCaP cells were treated with 0.01% DMSO, PEITC (2.5 or 5 μM), or 5-Aza (2.5 μM) and TSA (0.5 μM) for 5 days. The media containing the drugs was changed on day 3 and day 5. Cells were harvested on day 6 for all the experiments. A) Representative images from the methylation-specific PCR experiments. Genomic DNA was extracted from cells harvested on day 6, and the bisulfite conversion was subsequently performed. M: methylated, U: unmethylated. The relative intensity of the methylated and unmethylated bands was measured using ImageJ software, and the values are presented in the lower panel. B) The methylation pattern of the 16 CpG sites in the fragment of RASSF1A promoter was analyzed using bisulfite genomic sequencing. Solid circles indicate methylated CpG sites, and empty circles indicate unmethylated CpG sites. Ten representative clones from each of the 3 independent experiments were selected for analysis. C) The percentage of CpG methylation is presented. The methylation percentage was calculated from three independent experiments as the number of methylated CpG sites over the total number of CpG sites examined. D) The effect of PEITC on RASSF1A mRNA expression. Total mRNA was isolated and analyzed using quantitative real-time PCR. The data are presented as the mean ± SEM of 3 independent experiments. * P < 0.05 versus the control group. # P < 0.01 versus the control group.
Figure 2.
Effect of PEITC on DNMT gene and protein expression in LNCaP cells. Total mRNA was isolated and analyzed using quantitative real-time PCR. Total proteins were extracted and analyzed using western blot assays. The relative fold change of protein expression levels was calculated by dividing the intensity of the protein band by the intensity of the control protein band, and the values were normalized to β-actin levels using ImageJ. A) DNMT1 gene expression B) DNMT3A gene expression C) DNMT3B gene expression D) Representative Western blots images of DNMT3A and 3B protein expression. The representative blots are from the same image. The lanes have been rearranged from the same blot to be consistent with the increasing order of PEITC treatment as reported in all the figures. (Original blots have been provided in the supplemental information) E) Data are presented as the mean ± SEM protein expression levels of 3 independent experiments. * P < 0.05 versus the control group, # P < 0.01 versus the control group.
Figure 3.
Effect of PEITC on HDAC protein expression in LNCaP cells. Total proteins were extracted and analyzed using western blot assays. The relative fold change of protein expression levels was calculated by dividing the intensity of the protein band by the intensity of the control protein band, and the values were normalized to β-actin levels using ImageJ. A) Representative western blot images of HDAC1, 2, 4 and 6 protein expressions. The representative blots are from the same image. The lanes have been rearranged to be consistent with the increasing order of PEITC treatment as reported in all the figures. (Original blots have been provided in the supplemental information) B) Data are presented as the mean ± SEM protein expression levels of 3 independent experiments. * P < 0.05 versus the control group. # P < 0.01 versus the control group.
Figure 4.
Effect of PEITC on A) p21 gene expression. Total mRNA was isolated and analyzed using quantitative real-time PCR. B) Representative western blot images of p21, caspase-3, caspase-7, Bax and Cyclin B1 protein expression. Total proteins were extracted and analyzed using western blot assays. The relative fold change of protein expression levels was calculated by dividing the intensity of each protein band by the intensity of the control protein band, and the values were normalized to β-actin levels using ImageJ. C) Data are presented as the mean ± SEM protein expression of 3 independent experiments. D) Cell cycle progression in LNCaP cells treated with PEITC or 5-Aza and stained with propidium iodide (PI) was analyzed using flow cytometry with a Beckman Coulter Gallios Flow Cytometer. The legend presents the distribution of cells in the sub G₀, G₀/G₁, S and G₂/M phases of the cell cycle. E) Percentage of normal, necrotic and apoptotic cells as determined by flow cytometry of cells stained with PI/Annexin V-FITC. In all panels, the bottom left quadrant represents normal cells, the top right quadrant represents necrotic cells and the bottom right quadrant represents apoptotic cells. The control group is presented in the panel on the left, cells treated with 5 μM PEITC are presented in the center panel, and cells treated with 5-Aza and TSA are presented in the panel on the right. *, P<0.05, # P < 0.01 versus the control group.
Figure 5.
Effect of PEITC on A) LNCaP Colony Formation. LNCaP cells were seeded at 800 cells/well density in 6 well dishes. Cells were treated with PEITC (2.5μM, 5μM, 10μM) or 0.1% DMSO (control) every 3 days after seeding for two weeks. The cell colonies were stained with 5 mL 0.01% (w/v) crystal violet. The number and area of colonies containing more than 50 individual cells were counted by ImageJ. B) Number of colonies in different treatment groups. C) Relative total colony area in different groups. Data are presented as the mean ± SEM of 3 independent experiments, # P < 0.01 versus the control group.
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