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Green tea–induced epigenetic reactivation of tissue inhibitor of matrix metalloproteinase-3 suppresses prostate cancer progression through histone-modifying enzymes

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Abstract

Green tea polyphenols (GTPs) and their major constituent, epigallocatechin-3-gallate (EGCG), have been reported to demonstrate many interesting biological activities, including anticancer properties. Recent studies on prostate cancer provide strong evidence that epigenetic mechanisms are major players in the regulation of matrix metalloproteinases (MMPs) and their binding partner tissue inhibitor of MMPs (TIMPs) involved in prostate cancer progression. Here we demonstrate that GTP/EGCG mediate epigenetic reactivation of TIMP-3 that plays a key role in suppressing invasiveness and cancer progression. Treatment of human prostate cancer DUPRO and LNCaP cells with 10 μg/mL GTP and 20 μM EGCG induced TIMP-3 mRNA and protein expression. This transcriptional activation of TIMP-3 was associated with the decrease in the expression of both enhancers of zeste homolog 2 (EZH2) and its catalytic product trimethylation of histone H3 at lysine 27 (H3K27me3) repressive marks at the TIMP-3 promoter with an accompanying increase in histone H3K9/18 acetylation. In addition, GTP/EGCG treatment significantly reduced class I histone deacetylase (HDAC) activity/expression and EZH2 and H3K27me3 levels in prostate cancer cells. EGCG/GTP exposure also reduced MMP-2/MMP-9 gelatinolytic activity and abrogated invasion and migration capabilities in these cells. Silencing of EZH2 and class I HDACs strikingly increased the expression of TIMP-3 independent of DNA methylation. Furthermore, clinical trials performed on patients undergoing prostatectomy consuming 800 mg EGCG (Polyphenon E) up to 6 weeks and grade-matched controls demonstrate an increase in plasma TIMP-3 levels. A marked reduction in class I HDACs activity/expression and EZH2 and H3K27me3 levels were noted in GTP-supplemented prostate tissue. Our findings highlight that TIMP-3 induction, as a key epigenetic event modulated by green tea in restoring the MMP:TIMP balance suppresses prostate cancer progression.

1 | INTRODUCTION

A key step in the complex process of cancer progression and the establishment of metastasis is the degradation of the extracellular matrix (ECM) achieved by a range of proteases including the matrix metalloproteinases (MMPs), a family of 24 individual enzymes in humans.[1,2] MMPs and other cellular proteins, such as the ECM protein, integrins, and adhesion signaling receptors, are also involved in cell migration.[3,4] A substantial body of evidence indicates the involvement of gelatinases/type IV collagenases (MMP-2 and MMP-9) secreted by malignant cells in cancer cell invasion and metastasis.[5] The gelatinases (MMP-2 and MMP-9) are produced in a latent form (pro-MMP) that requires activation and are inhibited by a class of protein referred as tissue inhibitor of metalloproteinases (TIMPs). TIMPs are natural inhibitors of MMPs and are involved in diverse biological processes, including cell growth, tumor progression, apoptosis, invasion, metastasis, and angiogenesis.[6] Four different TIMP genes (TIMP-1, -2, -3, and -4) and proteins are identified in humans, and all four inhibit active forms of most MMPs but with different affinities, by binding to the active site of the enzyme. The balance between MMPs and TIMPs plays a critical role in ECM remodeling and turnover. Studies suggest that during tumorigenesis, the expression of TIMPs is downregulated, which ultimately results in an increase in MMP activity, facilitating cancer progression.[1-6] Given the importance of MMPs and TIMPs in regulating tumor progression, in recent years, intensive efforts have been taken to develop therapeutic strategies designed to inhibit MMP activity. Failure of most synthetic MMP inhibitors, such as Batimastat, Marimastat, Prinomastat, and so on, in clinical trials, had led to attempts to develop safe and effective therapeutic strategies focused on mechanism-based inhibition of MMPs.[7,8] A promising alternative strategy is to induce endogenous MMP inhibitors and tumor suppressor genes such as TIMPs and reversion-inducing cysteine-rich protein with Kazal motifs (RECK) to block MMP activity.[9]

Some of the common molecular abnormalities in cancer are epigenetic alterations. Epigenetic changes are reversible changes that include key processes of DNA methylation, chromatin modifications, nucleosome positioning, and alterations in noncoding RNAs.[10-12] Chromatin modifications, such as histone acetylation/deacetylation and chromatin-binding proteins, affect the local chromatin structure, and in concert with DNA methylation, regulate gene transcription. In particular, histone deacetylases (HDACs) are recruited by sequence-specific transcription factors to distinct promoter regions for silencing target genes. HDACs are frequently overexpressed in various human cancers, and their differential expression often correlates with poor prognosis.[13,14] Such epigenetic alterations play a significant role in cancer development and progression. Many of the genes that play a role in regulating cell growth, differentiation, signal transduction, DNA repair, and others that are implicated in tumor metastasis and angiogenesis undergo hypermethylation, suggesting that an epigenetic gene inactivation is a common event in the development of cancer.[10-13] Recent studies provide strong evidence that epigenetic mechanisms are major players in the regulation of the MMP-2 and MMP-9 as well as the genes involved in the regulation of TIMPs.[15-17] Polycomb group protein enhancer of zeste homolog 2 (EZH2) is a member of polycomb repressive complex 2 that mediates transcriptional silencing either by methylation of lysine 27 in histone H3 (H3K27me3) or by recruiting DNA methyltransferases (DNMTs) to its target genes.[18,19] Several studies suggest that EZH2 is overexpressed in a variety of tumors and directly correlates with invasion and metastasis of malignant cells.[18-20] Shin and Kim[21] reported that EZH2 plays a key role in regulating the activity of MMPs in prostate cancer cells by epigenetically repressing TIMP-2 and TIMP-3. Importantly, TIMP-3 loss has been shown to accelerate tumor invasion and inflammation in a mouse model of prostate cancer.[22]

Numerous epidemiological and experimental studies suggest that consumption of green tea polyphenols (GTPs) is inversely correlated with the incidence of prostate cancer.[23,24] GTPs and its major constituent, epigallocatechin-3-gallate (EGCG), exhibit the ability to suppress cancer cell growth, induce apoptosis, and inhibit tumor angiogenesis.[25,26] They have also been shown to inhibit gelatinases, which play pivotal roles in basement degradation and promote tumor metastasis.[2-5] But the molecular mechanism(s) underlying these effects remains largely unknown. It is likely that EGCG imparts its anticancer effects through many different mechanisms. One such mechanism involves EGCG as a potent inhibitor of DNMTs, which prevents the hypermethylation of newly synthesized DNA strands.[27,28] It is also known to inhibit class I HDACs, which in turn causes chromatin relaxation, facilitating gene transcription.[29,30] We and others have demonstrated the ability of GTPs to alter gene expression by epigenetic modifications, including DNA methylation and chromatin remodeling, resulting in re-expression of some tumor suppressor genes.[27-30] Thus, chemoprevention by green tea as a natural inhibitor of DNMTs and HDACs in prostate epithelial and/or neoplastic cells could be a promising approach with minimal side effects and toxicity. These findings indicate that GTPs and EGCG may induce epigenetic modulation of MMPs and their natural inhibitors, which may prove to be an important molecular mechanism of action in prostate cancer cells.

In the present study, we investigated the effects of GTPs on TIMP-3 expression in prostate cancer cells and deciphered their molecular mechanism of action. Our results demonstrate that GTPs and EGCG treatment could induce endogenous TIMP-3 mRNA and protein levels in human prostate cancer DUPRO and LNCaP cells. GTP-mediated TIMP-3 induction inversely correlated with EZH2 and class I HDACs protein levels. A detailed study into molecular mechanism revealed that treatment with GTP and EGCG decrease repressive chromatin mark H3K27me3 at the TIMP-3 promoter with a concomitant increase in transcriptionally active chromatin mark on histone H3 acetylated at lysine 9/18. Similar observations were noted on short-term supplementation of GTPs with a marked increase in TIMP-3 levels and a simultaneous decrease in class I HDACs, MMP-2/MMP-9, and EZH2 expression in patients with prostate cancer.

2 | MATERIALS AND METHODS

2.1 | Antibodies and reagents

Anti-TIMP-3 (SC-373839), anti-HDAC-1 (SC-7872), anti-HDAC-2 (SC-6296), anti-HDAC-3 (SC-11417), anti-HDAC-8 (SC-11405), anti-actin (SC-47778), anti-α-tubulin (SC8035), goat anti-mouse IgG-HRP (SC-2005), bovine anti-goat IgG-HRP (SC-2350), and goat anti-rabbit IgG-HRP (SC-2004) antibodies were purchased from Santa Cruz Biotechnology (Dallas, TX). Anti-H3K27me3 (mAb-Cam6002) was purchased from Abcam (Cambridge, MA); anti-acetyl H3K9/18 (07-593) was purchased from Upstate Biotechnology (Lake Placid, NY); and anti-Histone H3 (Clone A3S) and anti-EZH2 (07-689) were purchased from EMD Millipore, Billerica, MA.

2.2 | Cell culture and treatment

Androgen-responsive human prostate cancer LNCaP cells were obtained from American Type Culture Collection (Manassas, VA) and authenticated using short tandem repeat profiling. Androgen-refractory metastatic human prostate cancer DUPRO cells were obtained from Dr Rajvir Dahiya at the University of California at San Francisco with published reference and were not authenticated by the recipient. The cells were grown in RPMI-1640 supplemented with 10% fetal bovine serum (FBS), 50 U/mL penicillin and 50 μg/mL streptomycin in 100-mm tissue culture plates at 37°C in a humidified atmosphere (5% CO_2). The cells were plated at 20% seeding density and allowed to settle for 24 hours. Media was replaced and cells received the following treatment (optimum dose for each treatment was previously determined): 10 μM 5-azacitidine (5-aza; Sigma-Aldrich, St. Louis, MO), 40 ng/mL trichostatin A (TSA; Sigma-Aldrich), 5 μM 3-deazaneplanocin A (DZNep; Cayman Chemical, Ann Arbor, MI), 10 μg/mL Polyphenon E (Mitsui Norin, Japan), and 20 μM EGCG for indicated time periods. Polyphenon E is a standardized green tea extract composed of (−)-EGCG, 65%; (−)-epigallocatechin, 4%; (−)-epicatechin, 9%; (−)-epicatechin-3-gallate, 6%; (−)-gallocatechin-3-gallate, 4%; (−)-catechin-3-gallate, 0.2%; gallocatechin, 0.2%; catechins, 1.1%; and caffeine, 0.7%.[30] Polyphenon E, hereafter referred to as GTP,

and concentrations of 10 μg/mL Polyphenon E correspond to 14.0 μM of EGCG. For combined treatment with TSA and DZNep, the cells were cultured with TSA for 48 hours followed by the addition of DZNep for 24 hours. Every alternate day, the media was replaced with the fresh media and GTP or EGCG was replaced with a fresh dose.

2.3 | Study design and clinical specimens

Polyphenon E–supplemented prostate cancer specimens (n = 5) and blood samples were obtained from the clinical trial NCT01340599 that received a total of 800 mg EGCG daily as four capsules with a meal (total of 1.3 g Polyphenon E) during a 6-week interval between prostate biopsy and radical prostatectomy in men with localized disease. The time of Polyphenon E administration varied between patients and was subjected to their surgical schedules. Grade-matched prostate tissue (no treatment) and blood specimens were procured from another IRB approved study (08-03-38) from the University Hospitals Cleveland Medical Center.

2.4 | Transfection of cell packaging

Packaging cells 293T were plated in 100 mm plates with 20% cell density one day before transfection in RPMI containing 10% heat-inactivated FBS without penicillin-streptomycin. Six microgram of PLK0.1 (control vector only), shEZH2, shHDAC-1 (4814), shHDAC-2 (4818), shHDAC-3 (4826), shHDAC-8 (4849) along with second-generation packaging construct (pCMV-dR8.74), and pMD2G was transfected using lipid transfection (Lipofectamine/PLUS reagent; Invitrogen Corporation, Grand Island, NY) as per the protocol supplied by the manufacturer. Media was collected for two subsequent days and layered onto prostate cancer cells DUPRO and LNCaP to infect them with the virus after adding 10 μL of 4 mg/mL polybrene per 10 mL and sterilize passing through 0.45 μM microfilters.

2.5 | RNA extraction and reverse transcription–semi quantitative polymerase chain reaction

Total RNA was extracted from cell lines after appropriate treatment at indicated time points using TRIzol reagent. The quality and quantity of extracted RNA were determined using a NanoDrop spectrophotometer. Two micrograms of RNA from each sample was subjected to reverse transcription reaction using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Grand Island, NY). The TIMP-3 expression levels were assessed by semiquantitative using the primer set: forward primer - 5′-CTACCTGCCTTGCTTTGTGAC-3′ and reverse primer - 5′-ATCCTCG GTACCAGCTGCAG-3′ (amplicon size 140 bp). GAPDH was used as an internal control and the primers used were: forward primer 5′-CAACG GATTTGGTCGTATTGG-3′; reverse primer 5′-GCAACAATATCCACTTT ACCAGAGTTAA-3′. Each reaction was carried out in a reaction volume of 20 μL containing 100 ng cDNA synthesized from 2 μg RNA, 10 μL 2X polymerase chain reaction (PCR) master mix (Promega, Madison, WI) and forward and reverse primers (100 ng each in final reaction). The optimized PCR conditions for TIMP-3 and GAPDH amplification were

initial denaturation at 95°C for 5 minutes followed by 25 cycles of denaturation at 95°C for 30 seconds, annealing at 58°C for 20 seconds, and extension at 72°C for 30 seconds. Final extension was carried out at 72°C for 5 minutes followed by a hold at 4°C. PCR products were run in 2% ethidium bromide (EtBr)-Agarose gel at 110 V in TBE buffer and visualized in a UV transilluminator. Images of ethidium bromide stained bands were captured using a Kodak gel imaging system. For each gene, the band intensities were normalized against GAPDH. The normalized band intensities obtained for controls have been assigned the value of 1 or 100 and those obtained for various treatments were plotted as fold increase or percent with respect to control, respectively.

2.6 | Western blot analysis

Cells were lysed in the lysis buffer (Tris-HCl 50 mM, NaCl 150 mM, Triton X-100 1%, EGTA 1 mM, sodium pyrophosphate 20 mM, pH 7.4 containing protease inhibitors cocktail (10 μL/mL), NaF (10 mM), DTT (1 mM), PMSF (0.1 mM), and sodium orthovanadate (1 mM) on ice as previously reported.[30] Prostate tissue samples were homogenized using Bullet Blender Green Beads lysis kit loaded to a Bullet Storm homogenizer in refrigerated conditions (Midwest Scientific Inc, Valley Park, MO). After lysis, all samples were cleared by centrifugation at 15,000 rpm for 20 minutes. To confirm equal loading, the total protein concentration was determined using the BCA method (Bio-Rad, Hercules, CA). Proteins were resolved using SDS-PAGE and then transferred to nitrocellulose membrane. Non-specific binding sites on the membrane were blocked using 5% nonfat skimmed milk and incubated with the primary antibody followed by the incubation with a secondary antibody. Proteins were detected using BrightStar Femto HRP Chemiluminescent 2-Component Substrate ECL Kit (Alkali Scientific Inc, Fort Lauderdale, FL).

2.7 | Chromatin immunoprecipitation assay

Untreated and treated cells were incubated with 1% formaldehyde for 15 minutes at room temperature for cross-linking. The reaction was then terminated using a 0.125 M final concentration of glycine. After cross-linking, chromatin was digested using monococcal nuclease enzyme and incubated with EZH2 antibody, H3K27me3 antibody, H3K9/18 acetylated antibody overnight at 4°C. After reversing the cross-linking by incubating the samples at 65°C overnight, DNA was purified using phenol-chloroform-isoamyl reagent followed by ethanol precipitation. DNA was then dissolved in nuclease-free water. Immunoprecipitated DNAs, beads or input controls were subjected to PCR amplification for 30 cycles of the following cycling conditions: stage 1, 95°C for 5 minutes (1 cycle); stage 2, 95°C for 30 seconds and 60°C for 30 seconds and 72°C for 1 minute (30 cycles); and stage 3, 72°C for 7 minutes (1 cycle). Primers were used for human TIMP-3 gene for the region having YY1 binding site in the promoter region, as published before.[31] PCR products were subjected to electrophoresis on a 2% EtBr-Agarose gel and visualized under UV light.

2.8 | Wound healing assay

Cells were seeded at 70% to 80% confluence into six-well culture dishes. After the cells grew to confluence, using a sterile pipette tip, at least three scratch wounds per plate were made. The cell monolayer was washed once with 1X PBS to remove any floating cells. Afterward, the culture medium was replenished with a fresh dose of 10 μM 5-aza, 40 ng/mL TSA, 5 μM DZNeP, 20 μM EGCG, and 10 μg/mL GTP. The cells were incubated at 37°C in a 5% CO_2 humidified incubator and photographed at indicated time points. Cell migration areas were calculated using ImageJ software, as previously described.[31]

2.9 | Invasion assay

Transwell invasion assay was performed using 24-well ThinCert cell culture inserts from Greiner-Bio One (8-μM pore size Cat #662638 NC) to study the anti-invasive effect of GTP and EGCG on DUPRO cells harboring the empty vector backbone PLK0.1 and those silenced for EZH2 (shEZH2). The inserts were coated with 100 μL (1 mg/mL) Matrigel (Cat #3433-001-R1, TREVIGEN, Gaithersburg, MD). After coating, the inserts were incubated at 37°C in a CO_2 incubator for an hour before being used. Cell lines DUPRO PLK0.1 and DUPRO shEZH2-PLK0.1 were serum starved for 24 hours. Next day, the cells were trypsinized and washed with PBS and resuspended in serum-free media. The cells were counted, and a cell suspension (5×10^4 cells/mL) was added to the upper chamber containing the vehicle, 5 μM DZNep, 20 μM EGCG, and 10 μg/mL GTP. RPMI-1640 containing 10% FBS was placed in the lower chamber. The experiment was performed in triplicate. The cells were incubated for 48 hours in the CO_2 incubator at 37°C. After incubation, noninvasive cells and the gel in the upper compartment of the inserts were gently removed using a cotton swab moisturized with ddH$_2$O. The invasive cells in the lower chamber were washed twice with PBS and fixed with 3.7% formaldehyde for 2 minutes. After this, the cells in the lower chamber were washed again with FBS and permeabilized with methanol for 20 minutes. After permeabilization, the cells were washed again with PBS and stained with 0.5% crystal violet for 2 minutes. The wells were washed with PBS to remove the excess crystal violet and 1% Triton was added to each membrane. The chambers were incubated for 30 minutes, and the eluent was read at 480 nm using a plate reader. The difference in absorbance represented the effect of the compounds on the invading cells. The percentage of invasion was calculated and represented as bar graphs.

2.10 | Gelatin zymography

Culture supernatants were collected by centrifugation after each experiment and were then subjected to zymography on 7.5% SDS-PAGE gel copolymerized with 0.1% gelatin. The gel was washed in 2.5% Triton X-100 for 30 minutes (three times) to remove SDS and was then incubated overnight in activation buffer (10 mM Tris-HCl, pH 7, 5 mM $CaCl_2$, 1 μM $ZnCl_2$). After incubation, the gels were stained with 0.5% Coomassie Brilliant Blue followed by destaining

with methanol: glacial acetic acid: water (30:10:60) until clear bands were visible against the blue background.

2.11 | HDAC enzyme activity

HDAC enzyme activity was determined using 1X Epigenase HDAC Activity/Inhibition Direct Assay Kit (Cat #P-4034-48) obtained from EpiGenteK (Farmingdale, NY). HDAC assay was performed following the manufacturer's instructions measuring the color intensity at 450 nm.

2.12 | H3K27me3 measurement

H3K27me3 was determined using EpiQuik Trimethyl Histone H3K27 ELISA Kit (Cat #P-3112-96) obtained from EpiGenteK. H3K27me3 assay was performed following the manufacturer's instructions, measuring the color intensity at 450 nm.

2.13 | TIMP-3 ELISA assay

Plasma TIMP-3 levels were estimated using human the RayBio Human TIMP-3 ELISA kit (Cat #ELH-TIMP-3-1) purchased from Ray Biotech (Norcross, GA) as per the manufacturer's protocol.

2.14 | Statistical analysis

The digital images were quantified using the ImageJ software. The difference of each biomarker between control and treatment (green tea) was examined by two-tailed Student t test. TIMP-3, HDAC activity, and H3K27me3 data were summarized by mean and median (range) as well as bar plot and box plot. All tests were two-sided and the levels of significance designated as statistically significant are as follows *$P \le 0.05$, **$P \le 0.01$, and ***$P \le 0.001$.

3 | RESULTS

3.1 | GTPs suppress invasiveness and migration ability in human prostate cancer cells

To determine the effect of GTP and its major constituent EGCG on human prostate cancer cells, we examined the invasive and migration potential of the cells using transwell invasion and wound healing assays. As shown in Figure 1A, highly invasive DUPRO cells were treated with 20 μM EGCG and 10 μg/mL GTP, resulting in 80.1% and 84.6% decrease in invasiveness compared with untreated cells (100%) 48 hours after exposure. Similarly, in LNCaP cells, 20 μM EGCG and 10 μg/mL GTP treatment for 48 hours caused 89.0% and 92.8% decrease in invasiveness compared with untreated cells (100%). Combined treatment with 5 μM DZNep and 40 ng/mL TSA showed marked anti-invasive activity, causing 73.3% and 81.1% decrease in invasion ability in DUPRO and LNCaP cells after 48 hours of treatment. Exposure of cells to 5-aza, TSA, and DZNep alone resulted in 32% to

67% decrease in invasiveness in DUPRO cells and 44% to 76% decrease in LNCaP cells, respectively, 48 hours after treatment.

In cell migration assay, treatment of DUPRO cells with EGCG and GTP causes 20.7% and 11.2% wound closure, respectively, compared to untreated cells (92.5%) 24 hours after exposure. In LNCaP cells, 20 μM EGCG and 10 μg/mL GTP treatment for 48 hours caused 30% and 20% wound closure, respectively, compared with untreated cells (85.6%). Combined treatment with 5 μM DZNep and 40 ng/mL TSA showed marked antimigratory activity causing 25% and 31% wound closure in DUPRO and LNCaP cells after 24 and 48 hours treatment period. Upon exposure of cells to 5-aza, TSA, and DZNep alone, 20% to 93% wound area was occupied by DUPRO cells by 24 hours; whereas 18% to 77% wound area was occupied by LNCaP cells 24 to 48 hours after treatment (Figure 1B). Collectively, our data showed that 20 μM EGCG and 10 μg/mL GTP treatment decreased migration and invasiveness of human prostate cancer cells (Figure 1A and 1B).

3.2 | GTPs decrease MMP-2/MMP-9 activity in human prostate cancer cells

Since MMP-2 and MMP-9 (type IV collagenases/gelatinases) are prominent MMPs involved in ECM degradation and tumor cell migration that bind with TIMPs in 1:1 stoichiometry to regulate its activity, we next determined the enzymatic activity of these two MMPs in the conditioned media from the same treatment groups using gelatin zymography. EGCG and GTP treatment caused a significant reduction in gelatinolytic activity (MMP-2/MMP-9) in both DUPRO and LNCaP cells. As shown in Figure 2, in DUPRO cells, 20 μM EGCG treatment caused 55% decrease in MMP-2 and 86% decrease in MMP-9 enzymatic activity in the conditioned medium collected after 72 hours of treatment. Similarly, 10 μg/mL GTP treatment for 72 hours caused a 62% decrease in MMP-2 activity and 85% decrease in MMP-9 activity in DUPRO culture supernatant collected after 72 hours. In LNCaP cells, 66% decrease in MMP-2 activity and 39% decrease in MMP-9 enzymatic activity was observed after 20 μM EGCG treatment and 60% decrease in MMP-2 and 47% decrease in MMP-9 activity was observed after 10 μg/mL GTP treatment for 72 hours. In both cell lines, treatment with 5 μM DZNeP alone and in combination with 40 ng/mL TSA significantly reduced MMP-2/MMP-9 activity, whereas TSA alone was effective in suppressing MMP-2/MMP-9 activity in LNCaP cells only (Figure 2A and 2B).

3.3 | GTPs upregulate TIMP-3 levels in human prostate cancer cells

In prostate cancer, TIMP-3 silencing has been associated with epigenetic mechanisms involving aberrant histone modifications.[17,21] In our previous studies, we have demonstrated that EGCG and GTP are potent DNMTs and class I HDACs inhibitors and could reactivate epigenetically silenced genes such as GSTP1 in prostate cancer cells.[28] On the basis of the profiling of TIMP-3 mRNA expression levels in various prostate cancer cell lines, DUPRO and LNCaP cell lines exhibited significantly lower expressions of TIMP-3 compared
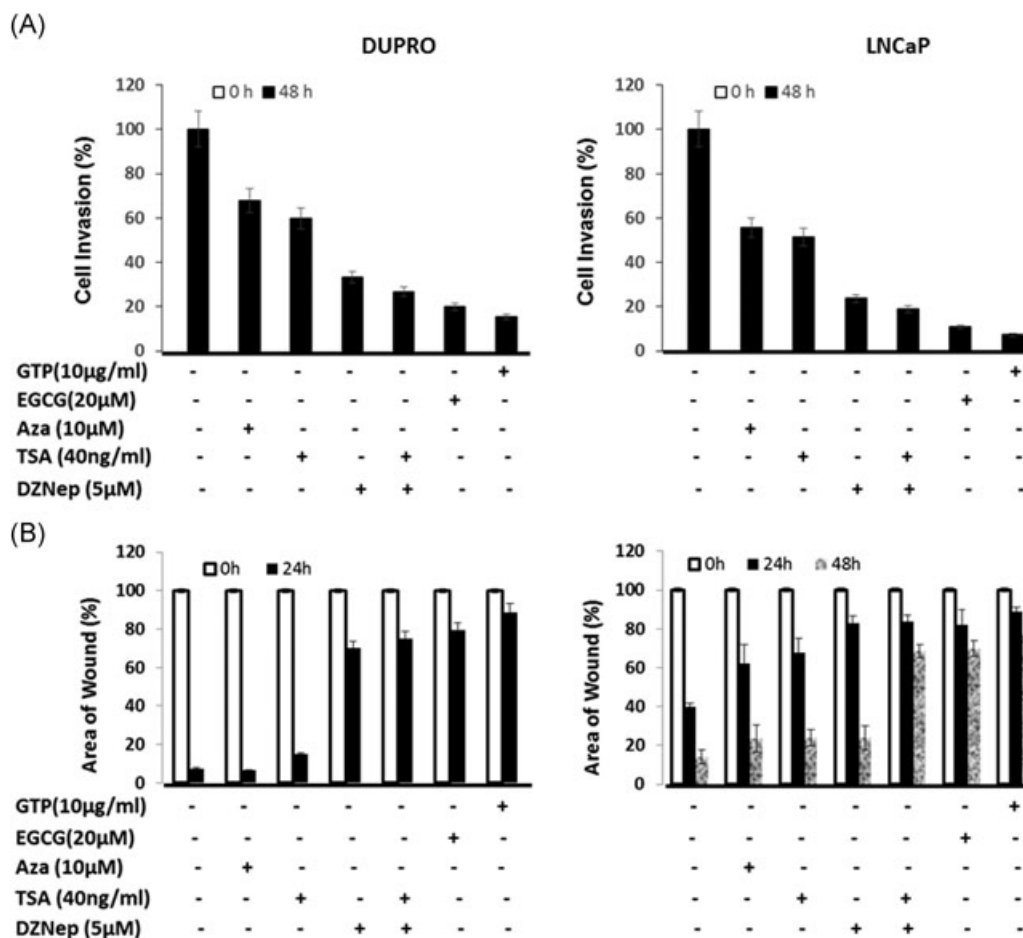
FIGURE 1 Effect of green tea polyphenol treatment on invasion and migration potential in human prostate cancer cells. A, Transwell invasion assay and (B) wound healing assay in DUPRO and LNCaP cells treated with 5-aza (10 µM), TSA (40 ng/mL), DZNep (5 µM), and combined DZNep (5 µM) + TSA (40 ng/mL), 20 µM EGCG, and 10 µg/mL GTP. Results were expressed as the percentage of the invading cells at 48 hours or difference in absorbance at 480 nm for invasion assay; whereas the remaining area determined by normalizing the area of wound after 24 or 48 hours, as indicated, to the initial wound area at 0 hours (set to 100%). Each bar represents the mean of 3 to 5 fields measured + SD. Two-tailed Student *t* test was used to compare treatment groups and control. Details are provided in the Section 2. 5-aza, 5-azacitidine; DZNep, 3-deazaneplanocin A; EGCG, epigallocatechin-3-gallate; GTP, green tea polyphenol; SD, standard deviation; TSA, trichostatin A

with nontumorigenic RWPE-1 cells and were hence chosen as in vitro models to evaluate the efficacy of EGCG and GTP treatment on TIMP-3 reactivation (data not shown). To ascertain the role of epigenetic mechanisms in TIMP-3 repression, DUPRO, and LNCaP cells were treated with various epigenetic modifying agents such as DNA demethylation agent, 5-aza; histone deacetylase inhibitor, TSA; and histone methyltransferase EZH2 inhibitor, DZNep. As shown in Figure 3, treatment with TSA and DZNep alone or in combination caused significant upregulation of TIMP-3 expression in both cell lines at mRNA and protein levels. No changes were observed in TIMP-3 expression after treatment with 5-aza in both cell lines. Exposure of DUPRO and LNCaP cells to a concentration of 20 µM EGCG and 10 µg/mL GTP for 72 hours causes a significant induction ($P < 0.05$) in TIMP-3 and mRNA levels (Figure 3A). These doses of EGCG and GTP are physiologically attainable and nontoxic to the cells for long treatment periods and have been previously used in our publications.[30-33] In DUPRO cells, 20 µM EGCG and 10 µg/mL GTP

treatment for 72 hours caused 3.6-fold and 3.3-fold increase in TIMP-3 mRNA levels, whereas in LNCaP cells, 2.2-fold and 2.3-fold upregulation in TIMP-3 expression was observed. Furthermore, a significant increase in TIMP-3 protein levels was also observed in both cell lines upon exposure to 20 µM EGCG and 10 µg/mL GTP for 3 to 7 days after treatment (Figure 3A-D).

3.4 | GTPs decrease class I HDACs and EZH2 protein levels in human prostate cancer cells

Histone modifications mediated by class I HDACs and histone methyltransferase EZH2 play an important role in regulating TIMP-3 expression in prostate cancer cells.[17,21] To determine if EGCG and GTP exposure affects the protein levels of class I HDACs (HDACs -1, -2, -3, and -8) and EZH2, we performed Western blot analysis on whole cell lysates of DUPRO and LNCaP cells treated with EGCG and GTP. Cells treated with other

epigenetic agents served as positive control. As shown in Figure 4A, exposure of DUPRO cells to 20 μM EGCG for 7 days caused a marked decrease in the protein levels of EZH2 by 90%, HDAC-1 by 23%, HDAC-2 by 87%, HDAC-3 by 86%, and HDAC-8 by 44%, respectively. Similarly, 10 μg/mL GTP treatment for 7 days in DUPRO showed a decrease in the protein levels of EZH2 by 17%, HDAC-1 by 26%, HDAC-2 by 73%, HDAC-3 by 89%, and HDAC-8 by 57%, respectively. In LNCaP cells, with respect to untreated cells, exposure to 20 μM EGCG for 7 days caused a decrease in the protein levels of EZH2 by 32%, HDAC-1 by 14%, HDAC-3 by 5%; whereas exposure to 10 μg/mL GTP for 7 days exhibited decrease in EZH2 levels by 10%, HDAC-1 by 54%, HDAC-3 by 5%, and HDAC-8 by 25%, respectively. The HDAC-2 protein levels remained unchanged as compared with untreated cells upon EGCG or GTP treatment. Similarly, no changes were observed in the HDAC-8 protein levels after EGCG treatment for 7 days in LNCaP cells; however, 3-day EGCG-treated LNCaP cells showed 7% decrease in HDAC-8 protein levels. Collectively, these results showed that EGCG- and GTP-mediated decrease in class I HDACs and EZH2 protein levels negatively correlates with TIMP-3 expression in human prostate cancer cells.

3.5 | Knockdown of class I HDACs and EZH2 upregulates TIMP-3 expression in prostate cancer cells

To confirm the regulation of TIMP-3, we used specific shRNAs for each target knocking down individually class I HDACs (HDACs -1, -2, -3, and -8) and EZH2 in both DUPRO and LNCaP cells. Interestingly, compared with only vector-treated cells, TIMP-3 protein levels were upregulated in all class I HDACs and histone methyltransferase EZH2 knockdown cells (Figure 4B). In DUPRO

cells, highest induction in TIMP-3 protein levels was observed in HDAC-8 and EZH2 knockdown cells whereas in LNCaP cells and an equivalent TIMP-3 induction pattern was noticed in all class I HDACs and EZH2 knockdown cells. Our results suggest that in human prostate cancer cells, TIMP-3 silencing is due to aberrant histone modifications mediated by class I HDACs and EZH2.

3.6 | EZH2 modulates the effect of GTPs in human prostate cancer cell invasion

In the next set of experiments, we determined the effect of GTPs on cell invasion after EZH2 knockdown in DUPRO cells. Knockdown of EZH2 resulted in decrease in overall invasiveness in these cells. Exposure of DUPROPLK0.1 cells with 20 μM EGCG and 10 μg/mL GTP treatment for 48 hours resulted in 28.1% and 38.6% decrease in invasiveness compared to untreated cells (100%) 48 hours after exposure. The anti-invasive effect of GTPs was more pronounced in DUPROshEZH2 cells where treatment with EGCG and GTP resulted in 76.7% and 90.3% decrease in invasiveness compared with untreated cells. A similar trend was noted in cells treated after DZNeP treatment (Figure 4C). Western blot analysis demonstrated decrease in the protein expression of EZH2 after EGCG and GTP exposure, with a concomitant increase in TIMP-3, whereas no significant change in the protein expression of MMP-2 and MMP-9 was noted posttreatment (Figure 4D). Our results demonstrate that the anti-invasive effect of EGCG and GTP is more pronounced in EZH2 knockdown condition compared with control vector cells (Figure 4C). These data indicate that the anti-invasive effect of GTPs depend on EZH2 expression, which in turn regulates TIMP-3 expression. However, the involvement of other molecular pathways modulated by GTPs cannot be ruled out.
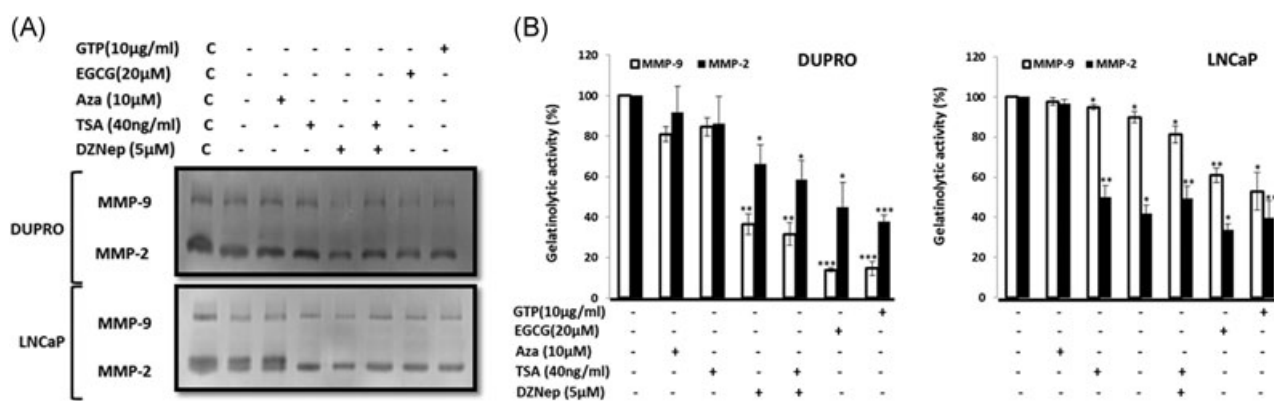

FIGURE 2 Effect of green tea polyphenol treatment on gelatinolytic activity (Pro-MMP-2 and Pro-MMP-9) in human prostate cancer cells. A, Gelatin zymography assay for MMP-2 and MMP-9 activity in DUPRO (top panel) and LNCaP (bottom panel) cells treated with 5-aza (10 μM), TSA (40 ng/mL), DZNep (5 μM), and combined DZNep (5 μM) + TSA (40 ng/mL), 20 μM EGCG, and 10 μg/mL GTP for 72 hours. Conditioned media (serum-free) was collected after 72 hours treatment and culture supernatant volume equivalent to 40 μg total protein was used in the assay. HT1080 cell line conditioned medium was used as a positive control and also served as a molecular weight marker. B, MMP-2 and MMP-9 band intensities (clearance zones) were quantified by densitometry using ImageJ software. Columns represent mean + SD of three independent assays. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ vs control. Details are provided in the Section 2. 5-aza, 5-azacitidine; DZNep, 3-deazaneplanocin A; MMP, matrix metalloproteinase; SD, standard deviation; TSA, trichostatin A
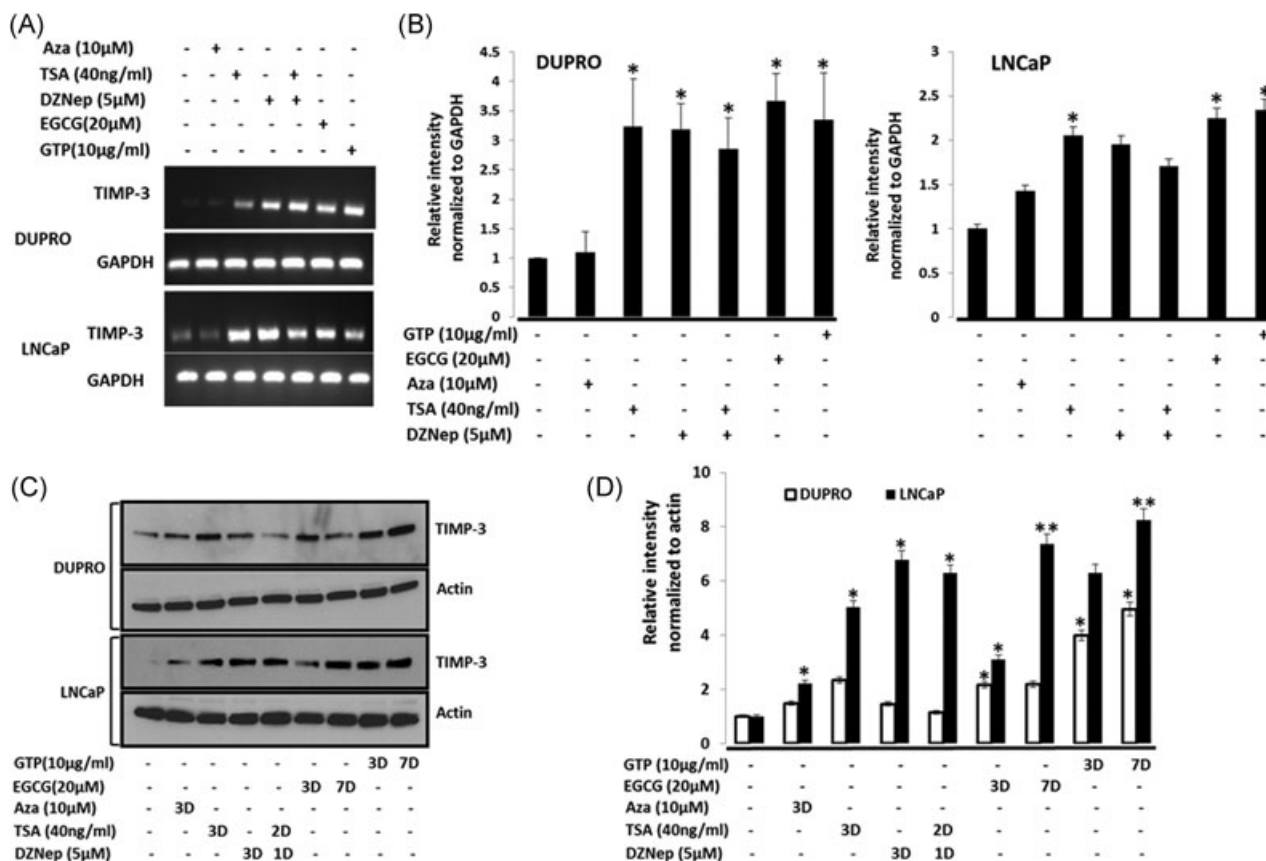
FIGURE 3 Effect of green tea polyphenols on induction of TIMP-3 in human prostate cancer cells. DUPRO and LNCaP cells were treated with 20 μM EGCG and 10 μg/mL GTP for 72 hours. Other epigenetic modifying agent treatments (as indicated) were used as positive controls. A, Change in TIMP-3 mRNA levels were detected by RT-PCR. GAPDH was used as internal control. B, Image analysis shows relative intensities of TIMP-3 bands normalized to GAPDH in DUPRO and LNCaP cells. Columns represent the mean TIMP-3 levels + SD of three independent experiments. Two-tailed Student t test was used to compare TIMP-3 expression levels between individual treatment groups and control. *$P < 0.05$ vs control. C, Western blot analysis of TIMP-3 protein levels. Forty micrograms of total protein were loaded in each lane and β-actin was used as loading control. D, Image analysis shows relative intensities of TIMP-3 bands normalized to β-actin. Columns represent the mean TIMP-3 levels + SD of three independent experiments. Two-tailed Student t test was used to compare TIMP-3 expression levels between individual treatment groups and control. **$P < 0.01$, ***$P < 0.001$ vs control. Details are provided in Section 2. EGCG, epigallocatechin-3-gallate; GTP, green tea polyphenol; PCR, polymerase chain reaction; SD, standard deviation; TIMP-3, tissue inhibitor of matrix metalloproteinases-3

3.7 | GTPs decreases H3K27 trimethylation and increases H3K9/18 acetylation in human prostate cancer cells

Next we determine the effect of EGCG and GTP treatment on histone modification marks catalyzed by EZH2 and class I HDACs in DUPRO and LNCaP cells. As shown in Figure 5A and 5B, Western blot analysis of acid-extracted histones from prostate cancer cells treated with 20 μM EGCG and 10 μg/mL GTP for 72 hours showed a marked decrease in repressive H3K27 trimethylation marks (histone H3 trimethylated at lysine 27). These results were further confirmed by H3K27 trimethylation ELISA assay (Figure 5C). A corresponding increase in H3K9/18 acetylation (histone H3 acetylated at lysine 9/18) was noted at the global level. In DUPRO cells, EGCG and GTP treatment resulted in 37% and 26% decrease in global H3K27 trimethylation, whereas in LNCaP cells, 46% and 84% decrease was noted posttreatment. In contrast, elevated levels of active H3K9/18 acetylation marks were observed in human prostate cancer cells

exposed to 20 μM EGCG and 10 μg/mL GTP for 72 hours. In DUPRO cells, EGCG and GTP treatment led to 88% and 103% increase in global H3K9/18 acetylation; whereas in LNCaP cells, the increase was found to be 50% and 101%, respectively. This data confirms that EGCG- and GTP-mediated decrease in class I HDACs and EZH2 may lead to an overall increase in transcriptionally active histone signature (H3K9/18 Ac) and reduction in repressive histone marks (H3K27me3) in human prostate cancer cells.

To address the effect of EGCG and GTP treatment on histone modification pattern specifically at the TIMP-3 promoter, we performed ChIP using specific primer set covering the YY1 binding site in TIMP-3 promoter[21] and targeting EZH2, H3K27me3, and H3K9/18 Ac histone marks. Treatment of DUPRO and LNCaP cells with 20 μM EGCG and 10 μg/mL GTP for 72 hours decreased EZH2 binding at the TIMP-3 promoter with a corresponding reduction in EZH2 catalyzed repressive H3K27me3 mark. In contrast, an increase in the enrichment of active H3K9/18 Ac mark at the TIMP-3 promoter was observed after EGCG and GTP treatment (Figure 5D).
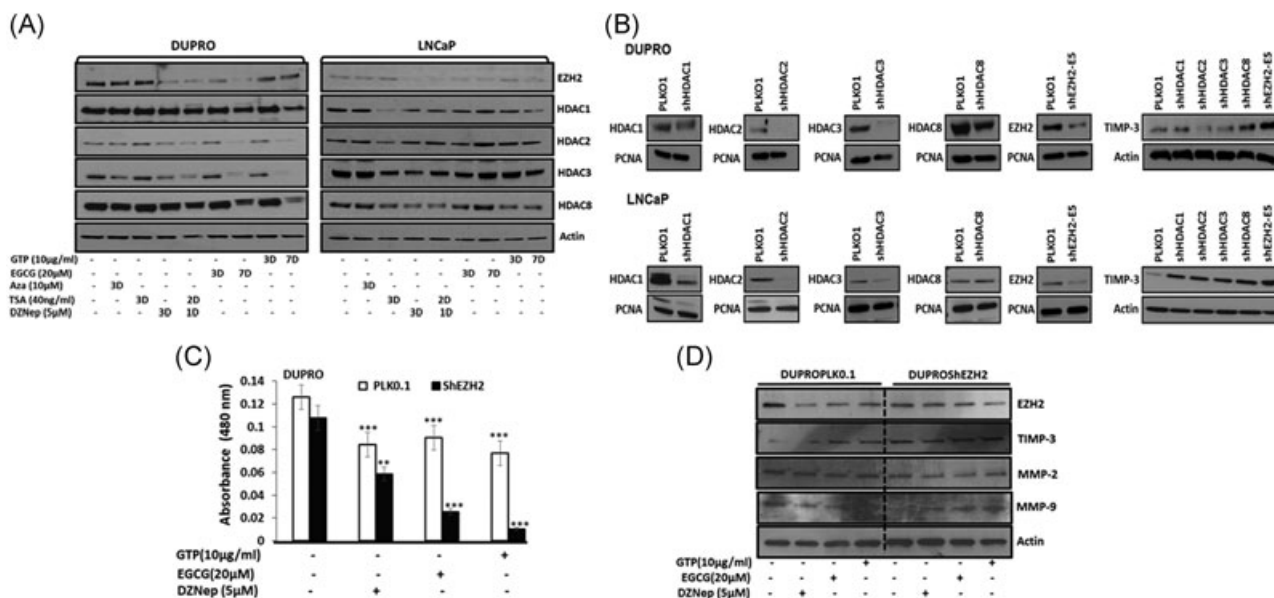
FIGURE 4 Effect of green tea polyphenols on class I HDACs and histone methyltransferase EZH2 protein levels in human prostate cancer cells. A, Western blot analysis of class I HDACs (HDAC-1, -2, -3, and -8) and EZH2 protein levels in DUPRO and LNCaP cells treated with 5-aza (10 μM), TSA (40 ng/mL), DZNep (5 μM), and combined DZNep (5 μM) + TSA (40 ng/mL), 20 μM EGCG and 10 μg/mL GTP for 3 days (3D) or 7 days (7D) as indicated. Forty microgram protein was loaded in each lane and β-actin was used as loading control. B, Effect of class I HDACs and EZH2 knockdown on TIMP-3 expression in prostate cancer cells. Western blot analysis of class I HDACs (HDAC-1, -2, -3, and -8) and EZH2 knockdown status in DUPRO (top panel) and LNCaP (bottom panel) generated using lentiviral vectors specific for EZH2 (shEZH2), HDAC-1 (shHDAC-1 4814), HDAC-2 (shHDAC-2 4819), HDAC-3 (shHDAC-3 4826), and HDAC-8 (shHDAC-8 4849). C, Effect of green tea polyphenols on cell invasion after EZH2 knockdown. DUPROPLK0.1 and DUPROshEZH2 cells were subjected to 5 μM DZnep, 20 μM EGCG, and 10 μg/mL GTP for 48 hours and transwell invasion assay was performed. Columns represent mean + SD of three independent assays. *$P < 0.05$ and ***$P < 0.001$ vs control. D, Effect of green tea polyphenols on DUPROPLK0.1 and DUPRO shEZH2 cells treated with 5 μM DZnep, 20 μM EGCG, and 10 μg/mL GTP for 3D. Western blot analysis for EZH2, TIMP-3, MMP-2, and MMP-9 using 40 μg protein loaded in each lane and β-actin was used as loading control. Details are provided in the Section 2. 5-aza, 5-azacitidine; DZNep, 3-deazaneplanocin A; EGCG, epigallocatechin-3-gallate; EZH2, enhancer of zeste homolog 2; GTP, green tea polyphenol; HDAC, histone deacetylase; MMP, matrix metalloproteinase; TIMP-3, tissue inhibitor of matrix metalloproteinases-3; TSA, trichostatin A

These findings were consistent with changes in overall histone methylation and acetylation levels and correlated with the induction of TIMP-3 expression in DUPRO and LNCaP cells treated with EGCG and GTP.

3.8 | Polyphenon E supplementation upregulate TIMP-3 levels, decreases MMP-2/MMP-9, EZH2 and class I HDACs expression in MEN with prostate cancer

Next we analyzed the plasma TIMP-3 levels along with HDAC activity and H3K27 trimethylation in the tissue lysate obtained from the patients supplemented with Polyphenon E and subjects without treatment (Figure 6A). There was a significant difference of TIMP-3 between control and treatment ($P < 0.0001$) with an elevated value of TIMP-3 in Polyphenon E group (0.292 ng/mL vs 0.166 ng/mL in control group). The HDAC activity and H3K27me3 also changed significantly in the tissue lysates from the Polyphenon E-treated group. A marked decrease was observed in the HDAC activity ($P < 0.003$) and H3K27me3 ($P < 0.0001$), compared with the no treatment group.

Higher expressions of MMP-2/MMP-9 are associated with metastatic disease and decreased survival of prostate cancer patients.[34] Our previous findings suggest that GTPs could decrease the levels of MMP-2/MMP-9 in the prostate of transgenic adenocarcinoma of the mouse prostate (TRAMP) mice,[35] prompting us to assess the effect of Polyphenon E on MMP-2/MMP-9 expression in tissue obtained from prostate cancer patients. There was a significant decrease in the expression of MMP-2/MMP-9 in Polyphenon E group compared with no treatment. The decrease in MMP-2/MMP-9 expression changed the balance between MMPs and TIMPs with a concomitant increase in TIMP-3 expression as observed in the plasma samples of the Polyphenon E–supplemented group (Figure 6B).

Next we determined the protein expression of class I HDACs and EZH2 in Polyphenon E–supplemented prostate cancer patients. Our previous studies have identified GTP having ability to decrease class I HDAC and EZH2 levels in human prostate and breast cancer cells.[30,31] As shown in Figure 6C, a marked decrease in class I HDAC (HDAC1-3, and 8) and EZH2 protein expression was noted after Polyphenon E supplementation, compared with no treatment. The expression of these proteins associated with decrease in HDAC
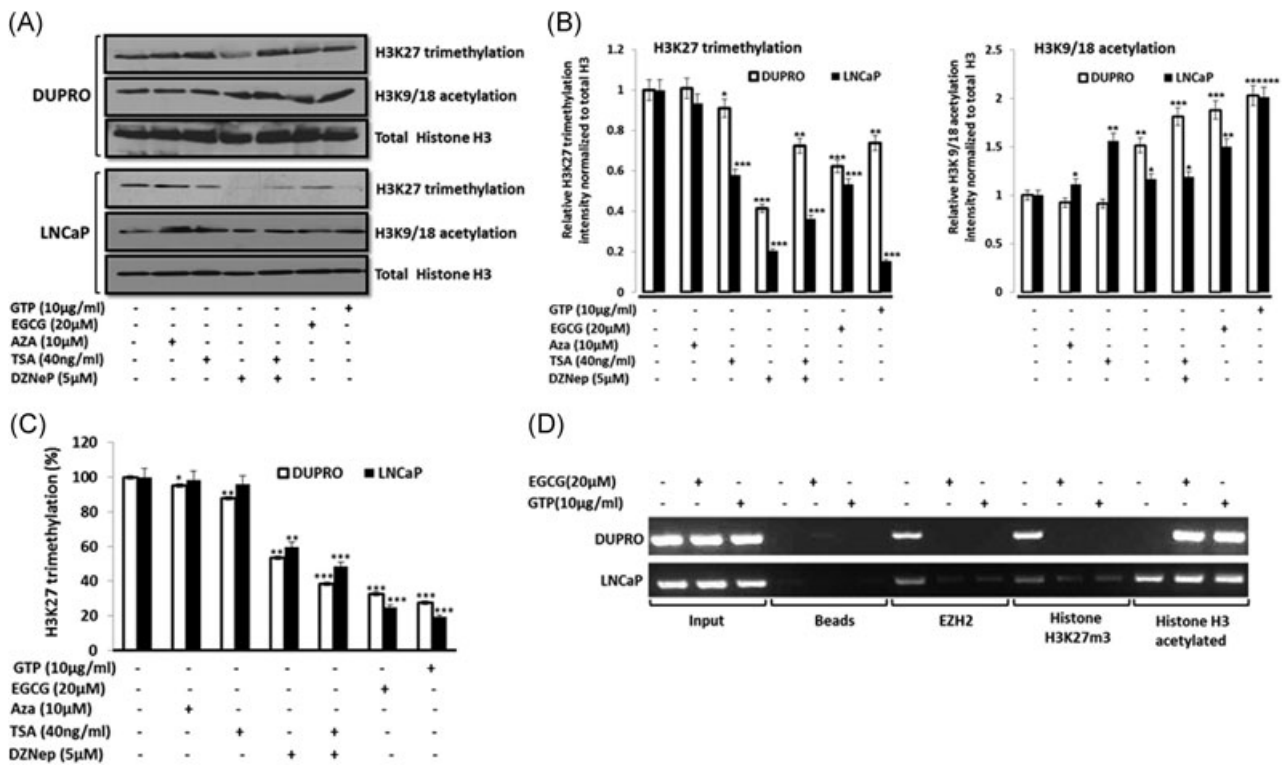
FIGURE 5 Effect of green tea polyphenols on H3K27me3 and H3K9/18 acetylation in human prostate cancer cells. A, Western blot analysis of acid-extracted total histones from DUPRO (top panel) and LNCaP (bottom panel) cells treated with 5-aza (10 μM), TSA (40 ng/mL), DZNep (5 μM), and combined DZNep (5 μM) + TSA (40 ng/mL), 20 μM EGCG, and 10 μg/mL GTP for 72 hours. Twenty microgram of total acid-extracted histone sample was loaded in each lane and total histone H3 served as internal control. B, Image analysis (right panel) shows ratio of relative intensities between histone H3 trimethylated at Lys 27 (H3K27me3) and histone H3 acetylated at Lys 9/18 (H3K9/18 Ac) bands normalized to total histone H3 in both cell lines. C, H3K27me3 by ELISA. D, Chromatin immunoprecipitation assay was performed to analyze the local histone modifications induced by 20 μM EGCG and 10 μg/mL GTP treatment for 72 hours at the TIMP-3 promoter. EGCG and GTP treatment caused decreased association of EZH2 and therefore less H3K27me3 enrichment at the TIMP-3 promoter with a concomitant increase in histone H3 acetylation in DUPRO (top panel) and LNCaP (bottom panel) cells. The experiment was repeated at least twice. Columns represent mean + SD of three independent assays. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ vs control. Details are provided in the Section 2. 5-aza, 5-azacitidine; DZNep, 3-deazaneplanocin A; EGCG, epigallocatechin-3-gallate; ELISA, enzyme-linked immunosorbent assay; EZH2, enhancer of zeste homolog 2; GTP, green tea polyphenol; SD, standard deviation; TIMP-3, tissue inhibitor of matrix metalloproteinases-3; TSA, trichostatin A

activity and H3K27 trimethylation observed in patients receiving Polyphenon E.

4 | DISCUSSION

Our studies show the reactivation of epigenetically silenced TIMP-3 by GTPs in prostate cancer cells and in prostate tissue obtained from patients with short-term supplementation of Polyphenon E. These results also provide convincing evidence that induction of TIMP-3 expression suppresses MMP-2/MMP-9 activity and tumor invasion in cell culture and clinical specimens. TIMPs are involved in the regulation of ECM and a balance between TIMPs and MMPs is critical for ECM degradation, a crucial step for tumor invasion and metastasis.[1-6] Absence of TIMP-3 has been shown to accelerate prostate tumor growth, proliferation, angiogenesis, and invasion in a murine model.[22] Consistent with the findings, human prostate cancer cell lines and tissues have reduced or undetectable levels of TIMP-3.[36,37] In clinical

specimens, TIMP-3 mRNA expression decreases with increasing Gleason score of prostate cancer.[37] Promoter DNA hypermethylation of TIMP-2 and TIMP-3 has been previously reported in clinical prostate cancer specimens. DNA hypermethylation in TIMP-3 promoter has been shown to occur at low frequency of approximately 6% to 21% in clinical specimens.[16,17] In our study, and accordingly, there was modest induction in TIMP-3 protein expression after treatment with demethylating agent 5-aza, which provides clues that other epigenetic modifications might be responsible for TIMP-3 silencing. Using two human prostate cancer cell lines viz. DUPRO and LNCaP as in vitro models, we observed that posttranslation modification of histone proteins play an important role in TIMP-3 gene silencing as knockdown of class I HDACs and EZH2 upregulates TIMP-3 expression in these cells.

Studies in prostate cancer implicate that EZH2 protein expression increase through successive stages of neoplastic transformation and malignant progression, and enhance EZH2 catalyzed H3K27me3 repressive mark results in the silencing of some key tumor
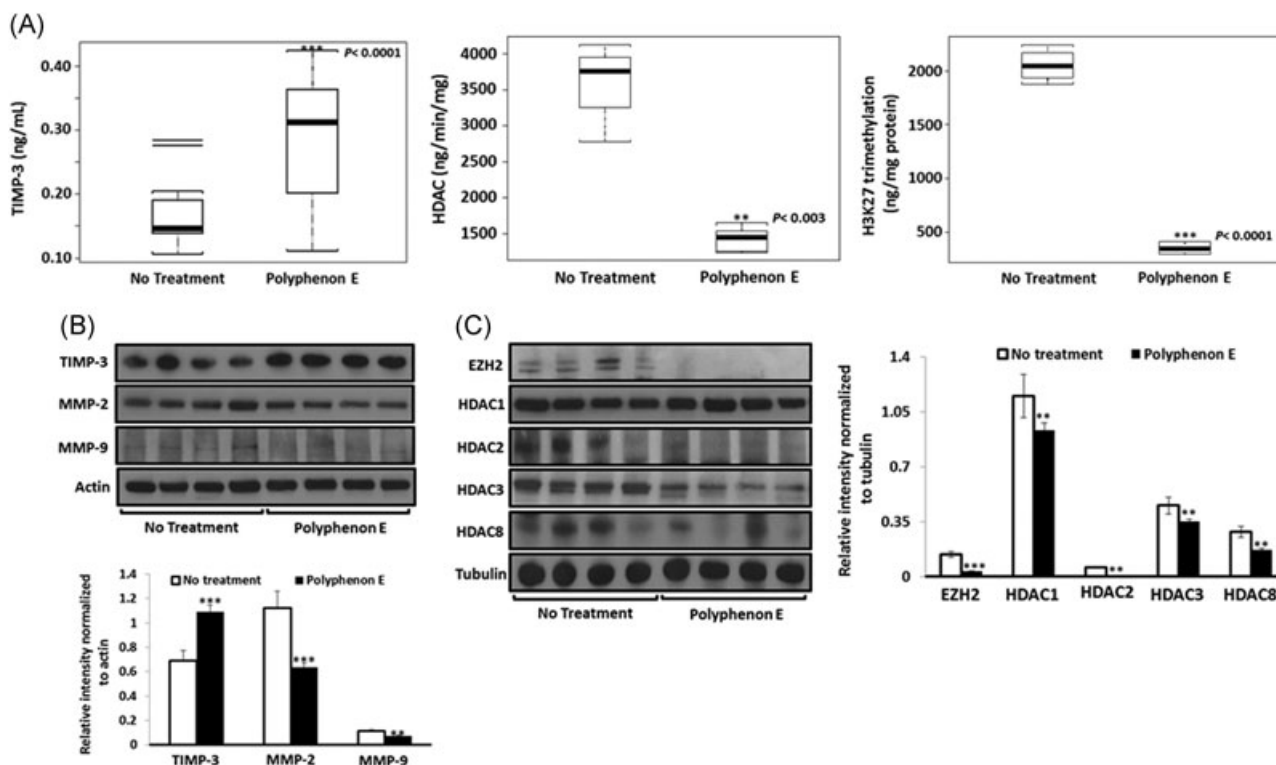
FIGURE 6 Effect of Polyphenon E supplementation on TIMP-3, HDACs, EZH2 and H3K27me3 in the plasma and tissue specimens obtained from prostate cancer patients and untreated controls. **(A)** Box plot of plasma TIMP-3 levels, HDAC activity and H3K27me3 in prostate tissue by ELISA assay. Black bar = median, box = 25th to 75th percentiles, Bars = entire range, Whisker line = outliers. The difference of each biomarker between control and treatment (Polyphenon E) was examined by t test. The test is two-sided and $P \leq 0.05$ was considered statistically significant. B, Western blot analysis of TIMP-3, MMP-2, and MMP-9 protein levels. Forty microgram of total protein was loaded in each lane and β-actin was used as loading control. Image analysis shows relative intensities of protein bands normalized to β-actin. C, Western blot analysis of EZH2 and class I HDACs (HDAC-1, -2, -3, and -8) protein levels. Forty microgram of total protein was loaded in each lane and tubulin was used as loading control. Image analysis shows relative intensities of protein bands normalized to tubulin. Two-tailed Student t test was used to compare protein expression between treatment group and untreated control. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ vs control. Details are provided in the Section 2. ELISA, enzyme-linked immunosorbent assay; EZH2, enhancer of zeste homolog 2; H3K27me3, trimethylation of histone H3 at lysine 27; HDAC, histone deacetylase; TIMP-3, tissue inhibitor of matrix metalloproteinases-3

suppressor genes.[18-20] Similarly, abnormally high activity of class I HDACs have been reported in prostate cancer, which has been linked to deacetylation and aberrant repression of several critical genes in prostate cancer.[38,39] Our studies showed that decreased TIMP-3 expression in prostate cancer DUPRO and LNCaP cells is associated with elevated EZH2 and class I HDAC levels. Chromatin immunoprecipitation studies further demonstrate that decreased EZH2 and repressive H3K27me3 chromatin modification and a corresponding increase in H3K9/18 Ac by combined DZNep and TSA treatment derepress TIMP-3 locus in both prostate cancer cell lines.

To our knowledge, this is the first study that provides a detailed mechanistic insight into the epigenetic induction of TIMP-3 by GTPs and its major constituent, EGCG in prostate cancer. We showed that GTP and EGCG treatment decreased EZH2 protein levels and hence its localization and catalytic activity, that is, H3K27me3 enrichment at the promoter region. Also, there was a significant decrease in HDAC-2, HDAC-3 and HDAC-8 levels in DUPRO and HDAC-1, HDAC-3 and HDAC-8 levels in LNCaP cells after treatment with GTP and EGCG. These observations are consistent with findings in the

clinical specimens receiving short-term supplementation of Polyphenon E. Suppression of class I HDACs might alter the balance resulting in simultaneous increase in the expression of histone acetyltransferases as evident by increase in H3K9/18 acetylation in both cell lines. This ultimately resulted in the deposition of increased transcriptionally active acetylated histone H3 at the TIMP-3 promoter. Thus, the present findings are in line with our previous studies which suggest that GTPs function as potent HDAC inhibitors mediated by enhancing the proteasome degradation of class I HDACs (HDAC-1, -2, -3, and -8) in prostate cancer cells.[30]

MMPs are key regulators of migration, invasion, metastasis, and classic hallmarks of cancer.[1-5] MMPs are a specific group of 24 enzymes whose catalytic activity is dependent on metal ions with potent ability to degrade structural proteins of the ECM.[2-4] Our studies demonstrate a significant decrease in MMP-2/MMP-9 activity, as measured by gelatin zymography, in conditioned media (serum-free) collected from EGCG/GTP-treated cells as a functional effect of TIMP-3 induction on DUPRO and LNCaP cells. A similar decrease in gelatinolytic activity was also observed in conditioned

media collected from DZNep and TSA-treated cells. EGCG has been shown to have multifunctional effect on MMP-2/MMP-9 including their expression, activation, secretion, and the induction of some inhibitors such as RECK.[40,41] On the basis of the previous studies, it is arguable that decrease in gelatinolytic activity might be due to direct effect of GTPs on MMP-2/MMP-9 expression.[42,43] A recent report on molecular docking analysis revealed a strong interaction between pro/active MMP-9 with GTPs, where galloyl group appears to be responsible for enhanced interaction.[26] These results corroborate with our previous findings in TRAMP mice where supplementation of green tea suppressed prostate tumor growth and complete inhibition of distant site metastasis.[44] Furthermore, GTP intake resulted in marked inhibition of markers of angiogenesis and metastasis most notably vascular endothelial growth factor, urokinase plasminogen activator, and MMP-2 and MMP-9 in these mice.[35,45] Similar results were observed in short-term clinical studies where Polyphenon E supplementation resulted in decreased MMP-2/MMP-9 expression, compared with the untreated group. Further in-depth studies are needed to define the extent of involvement of TIMP-3 on the regulation of MMP-2/MMP-9 activity and suppression of tumor cell invasion and metastasis.

The present results suggest that GTPs inhibits the migration and invasion of prostate cancer cells by reactivation of TIMP-3 and subsequent inhibition MMP-2/MMP-9 activity. In fact, the anti-invasive effects of GTPs was more pronounced in EZH2 knockdown cells, compared with vector control, providing evidence that EZH2 possess oncogenic potential facilitating invasion and migration capabilities. Importantly, EZH2-regulated pathways may not be the exclusive target of GTPs, rather other mechanisms such as epigenetic regulation of tumor suppressor genes might also play a key role.[27-33] Previously, the concentrations of EGCG in saliva and plasma have been reported as up to 4.8 μg/mL and 77.9 ng/mL after green tea consumption.[46,47] We have used 10 μg/mL GTP and 20 μM EGCG which are physiologically attainable concentration and corresponds to drinking 4 to 6 cups of green tea by an adult individual.[35,44] Epidemiologic and case-control studies provide some clues that regular consumption of green tea could decrease the risk of prostate cancer invasion and/or progression.[23-25] A clinical study conducted on high-risk subjects of prostate cancer consuming 600 mg GTP or a placebo for a year showed 3% incidence of prostate cancer in GTP group compared with 30% incidence in the placebo group.[48] Follow-up on these subjects after a year demonstrated that inhibition in prostate cancer was long lasting and 80% of subjects were still undiagnosed for cancer[49]; however, these studies do not provide insight into the molecular mechanism(s) related to tumor inhibition. Our studies on short-term supplementation of Polyphenon E at a dose of 800 mg/day for up to 6 weeks to patients undergoing radical prostatectomy provide mechanistic insight as higher levels of TIMP-3 and decrease expression of MMP-2/MMP-9 achieved through downregulation of EZH2 and class I HDACs play an important role as observed in cell culture studies. Polyphenon E supplementation at 800 mg/day in prostatectomy tissue were previously shown to result in low to undetectable levels of EGCG.[50] On the basis of our present findings, TIMP-3 can be used as a surrogate marker of GTP and EGCG response and warrants further investigation through well-designed clinical trial.

On the basis of our results, we conclude that the in vitro and in vivo anti-invasive effects of EGCG/GTP reported in prostate cancer may be due to, in part, through epigenetic induction of endogenous MMP inhibitor, TIMP-3. Also, our results provide the first detailed insight into the epigenetic regulatory mechanism causing TIMP-3 repression in prostate cancer, highlighting the role of histone-modifying enzymes EZH2 and HDACs. We demonstrated that GTP- and EGCG-mediated decrease in EZH2 and class I HDACs levels caused a corresponding global decrease in repressive chromatin mark H3K27me3 and an increase in transcriptionally open chromatin, that is, H3K9/18 Ac, respectively, in prostate cancer cells. This coincides with decreased EZH2 localization and H3K27me3 enrichment at the TIMP-3 promoter with a concomitant increase in H3K9/18 Ac, after GTP and EGCG treatment. Epigenetic induction of TIMP-3 may be one of the key mechanisms by which GTPs shift the MMPs: TIMPs balance toward TIMPs and inhibit active MMPs in prostate cancer. Additional studies are required to fully elucidate the molecular mechanisms by which GTPs, and EGCG in particular, inhibit tumor invasion and metastasis.

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CONFLICT OF INTERESTS

The authors declare that they have no conflict of interests.

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