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Effects of genistein supplementation on genome-wide DNA methylation and gene expression in patients with localized prostate cancer

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Abstract. Epidemiological studies have shown that dietary compounds have significant effects on prostate carcinogenesis. Among dietary agents, genistein, the major isoflavone in soybean, is of particular interest because high consumption of soy products has been associated with a low incidence of prostate cancer, suggesting a preventive role of genistein in prostate cancer. In spite of numerous studies to understand the effects of genistein on prostate cancer, the mechanisms of action have not been fully elucidated. We investigated the differences in methylation and gene expression levels of prostate specimens from a clinical trial of genistein supplementation prior to prostatectomy using Illumina HumanMethylation450 and Illumina HumanHT-12 v4 Expression BeadChip Microarrays. The present study was a randomized, placebo-controlled, double-blind clinical trial on Norwegian patients who received 30 mg genistein or placebo capsules daily for 3-6 weeks before prostatectomy. Gene expression changes were validated by quantitative PCR (qPCR). Whole genome methylation and expression profiling identified differentially methylated sites and expressed genes between placebo and genistein groups. Differentially regulated genes were involved in developmental processes, stem cell markers, proliferation and transcriptional regulation. Enrichment analysis suggested overall reduction in MYC activity and increased PTEN activity in genistein-treated patients. These findings highlight the effects of genistein on global changes in gene expression in prostate cancer and its effects on molecular pathways involved in prostate tumorigenesis.

Introduction

Prostate cancer is the most commonly diagnosed malignancy and the second leading cause of cancer death among men in the United States. It is estimated that approximately 180,890 new cases of prostate cancer and 26,120 deaths from prostate cancer occurred in the USA in 2016 (1). The common risk factors for prostate cancer are age, race/ethnicity, geography, family history and lifestyle (2). Depending on the severity of the disease, current treatment options for prostate cancer include single or a combination of therapies such as active surveillance, surgery, radiation therapy, chemotherapy, hormone therapy or vaccines (3). Although these interventions have significantly improved the quality of life of the patients and the overall survival rates, effective treatment of prostate cancer is still limited due to the major challenges such as genetic heterogeneity, tumor recurrence (~30% of the cases) and resistance to conventional chemotherapeutic drugs (4-6). Therefore, it is crucial to develop novel preventive and therapeutic strategies that have the potential to improve outcomes for prostate cancer patients.

Epidemiological studies have shown that there is a significant disparity in incidence and mortality rates of prostate cancer among different countries, with the highest rates in the USA and European countries and the lowest rates in Asian countries such as Japan and China (7,8). This wide variability in the prostate cancer rates across countries suggests that several factors including genetic, epigenetic and environmental differences play a key role in the etiology of the disease. Notably, it has been shown that Asian immigrants in the USA have an increased incidence of prostate cancer compared to those individuals with the same genetic background who live in Asia, indicating that environmental factors, especially the
diet, are major determinants of prostate cancer incidence (9). One of the remarkable dietary differences between Asian and Western countries is the amount of soy-based food consumption. Asian populations consume high quantities of soy food which is rich in isoflavones (~2 g of isoflavones per kg of fresh soybean) (10). It has been shown that plasma and prostatic fluid concentrations of isoflavones in Asian men are 10 to 100 times higher than those in Western men, with particularly high levels of the isoflavone genistein (11,12). An increasing body of population-based studies has demonstrated that high intake of soy isoflavones are associated with a 25-30% reduced risk of prostate cancer (13,14).

As the major biologically active isoflavone in the soy diet, genistein has been extensively investigated for its chemopreventive potential in various types of cancer, including prostate cancer. The average daily intake of genistein in Asian populations has been shown to be 20-80 mg whereas it is 1-3 mg in the USA, supporting the protective effects of genistein against prostate cancer in Asian men (15). Genistein reaches plasma concentrations of 1-5 μM 6-8 h after intake of soy-rich diet (11,16). The plasma half-life of genistein has been reported as 7.9 h in adults. In addition, concentrations of total soy isoflavones in prostate tissue have been shown ~6-fold higher than serum levels of isoflavones (17). Safety and pharmacokinetic studies of soy isoflavones have demonstrated that minimal clinical toxicity was observed in healthy subjects administered with purified soy isoflavones at doses that exceed normal dietary intakes (18).

Due to its structural similarity to the steroid hormone 17β-estradiol, genistein binds to estrogen receptors, ER-α and ER-β, with a higher affinity to ER-β, and acts as a natural selective estrogen receptor modulator (16,19,20). Genistein exerts its inhibitory effects on prostate cancer cells by upregulating the expression of ER-β, which has anti-proliferative and pro-apoptotic roles in prostate cells (21,22). In addition to its estrogenic activities, genistein regulates androgen receptor (AR)-mediated pathways in prostate cancer (23,24). Of note, it has been shown that the inhibitory effect of genistein on AR expression is also mediated by ER-β (25). Several other molecular mechanisms underlying the preventive effects of genistein on prostate cancer include the inhibition of cell proliferation by inducing G1 and/or G2/M cell cycle arrest (26-28), angiogenesis (29,30) and metastasis (31-33) and induction of apoptosis (34,35). Genistein exerts its pleiotropic effects in the context of prostate cancer through modulation of several cell signal transduction pathways such as IGF-1 (36), TGF-β (37), Wnt/β-catenin (36), NF-κB (38), AKT and MAPK (39) signaling. This modulation could be by direct binding to nuclear receptors or modification of the phosphorylation state of signal transduction proteins. In addition, genistein inhibits tyrosine kinase activities (40) and shows antioxidant properties (41,42) in prostate cells. Swami et al (43) demonstrated that genistein reduces prostate cancer progression by inhibiting prostaglandin synthesis and activity. Genistein has also been reported to have possible effects on DNA damage and repair in prostate cancer cells (42). Moreover, genistein inhibits DNA methylation (44-48) and histone modifications (47,48) and regulates miRNAs (49-52) in prostate cancer. It is of interest that genistein has been shown to enhance the efficacy of radiotherapy and chemotherapy (53,54).

Although numerous in vitro and in vivo studies have been conducted to understand the protective effects of genistein against prostate cancer demonstrated by epidemiological studies, the molecular mechanisms that govern how genistein affects the pathogenesis of prostate cancer still remain elusive. It is noteworthy that a major challenge is the wide variability of the effects of genistein depending on the dose, the form of administration, or the timing and duration of exposure (55). Despite the wealth of studies performed in human cell lines and animal models, only a few prospective randomized clinical trials have been conducted to examine the molecular effects of genistein on prostate cancer. In the present study, to the best of our knowledge for the first time, we investigated the effects of genistein intervention on global methylation and gene expression patterns in patients with localized prostate cancer, and identified novel targets that are differentially modulated by genistein supplementation, providing further mechanistic insights into the effects of genistein on prostate carcinogenesis.

Materials and methods

Subjects. Prostate specimens from a clinical trial of genistein supplementation prior to prostatectomy (56) were analyzed for global changes in DNA methylation and gene expression. Participants were recruited from the outpatient clinic at the Department of Urology, Oslo University Hospital, Oslo, Norway between April 2007 and August 2008. The study was approved by the Norwegian Medicines Agency, the Regional Ethics Committee, the Privacy Ombudsman and the Prostate Biobank at the Oslo University Hospital, Akers.

Genome-wide methylation profiling. Total DNA was isolated from frozen prostate tissues using DNase Blood and Tissue kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. DNA was submitted to the Emory Integrated Genomics Core for DNA methylation analysis using Illumina HumanMethylation450 BeadChip Microarrays. Methylation data are available on GEO (accession number GSE84749).

Genome-wide expression profiling. Total RNA was extracted from frozen prostate tissues using the mirVana miRNA Isolation kit (Life Technologies, Grand Island, NY, USA), followed by RNA clean-up using the RNeasy Mini kit (Qiagen). Total RNA was submitted to the Emory Integrated Genomics Core for gene expression analysis using the Illumina HumanHT-12 v4 Expression BeadChip Microarray. Microarray data are available on GEO (accession number GSE84748).

Quantitative PCR (qPCR) analysis. RNA was reverse-transcribed into cDNA using iScript cDNA Synthesis kit (Bio-Rad Laboratories, Hercules, CA, USA). Primers were designed using Primer3 tool. Sequences of the primers are listed in Table 1. qPCR was performed using iQ SYBR-Green Supermix (Bio-Rad Laboratories) on a Bio-Rad iCycler according to the manufacturer's protocols. Human β-actin gene, which has been shown to be a valid reference gene for normalization of qPCR in human tissue samples of prostate cancer, was used as an internal control in the present study (57). Normal prostate...
tissue sample was used as the calibrator. The relative changes in gene expression data were analyzed by the $2^{-\Delta\Delta CT}$ method. Triplicates were run for each sample. Data are presented as the mean ± standard deviation.

**Data analysis.** Gene expression analysis was performed using GenePattern ComparativeMarkerSelection module (58) comparing genistein-treated tumors to placebo-treated tumors. Illumina Microarray data were filtered to include genes that were detected ($P<0.05$) in at least one experimental group to result in a dataset of 15918 genes for analysis. The comparative marker selection module of GenePattern was used to compute two-sided Student’s t-tests between groups with 10,000 permutations to compute false discovery rates. The random seed used was 779948241. Hierarchical clustering was performed using Cluster software (59) and Java TreeView (60). Methylation microarray analysis was performed in R using CpGassoc module in Bioconductor (61). Data from the 450K probes was filtered to those in which the maximum - minimum $\beta$-value was >0.2 to result in 160K probes for differential methylation analysis. CpGassoc was used to identify 162 significant probes that were differentially methylated. Three probes were differentially methylated between genistein-treated tumor samples and placebo-treated tumor samples, three probes were significant between genistein-treated tumor samples and normal samples and 156 were significantly different between placebo-treated tumor samples and normal samples.

**Statistical analysis.** Mann-Whitney U test (two-tailed) was used to determine significant differences between two groups of data. $P<0.05$ was considered as statistically significant.

**Results**

**Clinicopathological characteristics.** We analyzed prostate tissue samples from a previous study, which was a randomized, placebo-controlled, double-blind Phase 2 clinical trial on Norwegian patients with localized prostate cancer who received 30 mg synthetic genistein or placebo capsules.

**Table I. Sequences of the primers used in the quantitative PCR analysis.**

| Primer name | Primer sequence (5’→3’) |
|-------------|-------------------------|
| CKS2-FP     | TTAGTCTCCGGCGAGTGTGTGT  |
| CKS2-RP     | CATAACATGCCGGTACTCTGT  |
| JAG1-FP     | AGTCGTGCATGCTCCAATCG   |
| JAG1-RP     | CCCACACACCTTGGCTC      |
| NOTCH3-FP   | GATGGTGACAGTGCTGGTG    |
| NOTCH3-RP   | CAGGGATGTTTGGGGGTC     |
| MMP26-FP    | GGACTTCTTTGAGGGCTATTTCCA |
| MMP26-RP    | GGAGGTTGCGGACCCCATCAG  |
| HIF1A-FP    | CACCCAGGCAGTAGGGATTG   |
| HIF1A-RP    | CTGCTGAATAATACACACCTACA |
| CDK6-FP     | GCTGACCAGCAGTAGCAGA    |
| CDK6-RP     | GCACACATCAAACACACCTGAC |
| CD24-FP     | CGCGGACCTTTCCTTTTGGGG |
| CD24-RP     | ACTGGAAATATTGCGTGGGTT |
| AMACR-FP    | CCGTTCTTGCTATGGGTC    |
| AMACR-RP    | AGCCTTGGATTTTCGCCTG    |
| MYC-FP      | CCTACCTCCTCAAGGACAGC  |
| MYC-RP      | TTGTTCTCCTCTCAGAGTCGC |
| SPP1-FP     | CAAACCCGCAGCAAGAAAA   |
| SPP1-RP     | GGCACAGACGATCTGGGTATT |
| NEU1-FP     | CGCAGCTATGATGCCGGTGA  |
| NEU1-RP     | GTGCAGGTTTCACTCGGAATCT |
| ADCY4-FP    | CCGGGACCCAGGTGCTCAT   |
| ADCY4-RP    | CAAGATACAGGCCGAGGACC  |
| $\beta$-actin-FP | CACAGACCTCGCCTTTGCCC |
| $\beta$-actin-RP | TGACCCATGCCCCACATCAC |
daily for 3-6 weeks before radical prostatectomy (56). The clinical and pathological characteristics of the cases were previously described (56). The availability of frozen tissue limited the sample size in this study and we investigated the DNA methylation and gene expression levels of prostate tumor samples from 10 patients who received genistein and 10 patients who received placebo. Four adjacent normal prostate tissue samples were also analyzed. Clinical data for the 20 patients analyzed here are provided in Table II. There were no statistically significant differences in age, levels of serum PSA and Gleason score between the two treatment groups.

Differential methylation in genistein-treated tissue compared with placebo-treated tissue. The genome-wide DNA methylation profiles of a total of 24 prostate samples from tumor or normal tissues were generated using Illumina HumanMethylation450 BeadChip kit. Methylation status of each sample was analyzed for 485,577 sites, covering 21,231 genes. We compared the methylation profiles of genistein-treated tumor samples with placebo-treated cases. In general, methylation changes were modest, and there was no significantly differentially methylated gene after correction for multiple hypothesis testing. However, uncorrected \( P \)-values indicated that \( RBM28 \) and \( CYTSB \) genes were demethylated in genistein-treated tumor samples compared to placebo-treated samples. The lack of statistical significance was likely due to the small numbers of samples analyzed in this study. We did observe 156 probes with significantly increased methylation in placebo-treated tumor tissues vs. normal tissues that were not significant between genistein-treated tumor tissues and normal tissues, suggesting that genistein may have had some demethylation effects (available upon request). These 156 probes corresponded to at least 92 separate genes including \( ADCY4 \), \( ALOX12 \), \( HAAO \), \( LRRC4 \), \( NEU1 \), \( RAPGEFL1 \) and \( WNT7B \) (Table III).

Gene expression profiling changes after genistein treatment. To identify molecular effects of genistein on mRNA levels in prostate cancer, we compared gene expression profiles of genistein-treated tumors with placebo-treated samples. Once again, there were no differentially expressed probes that remained statistically significant after correction for multiple hypothesis testing. However, there were 628 probes that reached nominally significant \( P \)-values (available upon request). Hierarchical clustering of this dataset showed strong segregation of patients with and without genistein treatment (Fig. 1). The genes with nominally significant \( P \)-values included \( NOTCH3 \), \( JAG1 \), \( CK52 \), \( HIF1A \), \( CDK6 \), \( MYC \), \( CD24 \), \( AMACR \), \( MMP26 \) and \( SPP1 \) genes (Table IV). \( NEU1 \) and \( ADCY4 \) did not reach nominal significance but had a trend towards significance, and integration of the methylation data with the paired gene expression profiling data indicated decreased methylation status and increased expression levels of \( ADCY4 \) and \( NEU1 \) genes in genistein-treated cases.

Validation of microarray data. We investigated the expression levels of 12 selected genes (Table IV) in all 24 samples analyzed
### Table III. List of 156 differentially methylated probes (92 genes).

| Target ID   | Gene name          | P-value (GT vs. PT) | P-value (GT vs. N) | P-value (PT vs. N) |
|-------------|--------------------|---------------------|--------------------|-------------------|
| cg00353923  | LRRC4; SND1        | ns                  | ns                 | 0.000214451       |
| cg00420348  | EFCAB4A            | ns                  | ns                 | 0.000247793       |
| cg00459232  | CD9                | ns                  | ns                 | 0.000270319       |
| cg00494665  |                   | ns                  | ns                 | 0.000274219       |
| cg00506168  | PDXK               | ns                  | ns                 | 0.000515556       |
| cg00578638  | RAPGEPFL1          | ns                  | ns                 | 3.67E-05          |
| cg01224366  | PDXK               | ns                  | ns                 | 0.000393857       |
| cg01228355  | CORIN              | ns                  | ns                 | 0.000881032       |
| cg01233722  | NFATC4             | ns                  | ns                 | 1.51E-05          |
| cg01398859  |                   | ns                  | ns                 | 0.000942104       |
| cg01561916  | HAAO               | ns                  | ns                 | 0.0015216         |
| cg01684881  | FZD2               | ns                  | ns                 | 0.000472597       |
| cg01856645  | DMGDH; BHMT2       | ns                  | ns                 | 0.000876054       |
| cg02072400  |                   | ns                  | ns                 | 3.73E-05          |
| cg02131967  | ACE                | ns                  | ns                 | 0.000468338       |
| cg02215070  | AKR1B1             | ns                  | ns                 | 0.000607743       |
| cg02493798  | ALOX12             | ns                  | ns                 | 0.000106934       |
| cg02534363  | NBEAL2             | ns                  | ns                 | 0.000263128       |
| cg02659920  | EPSL2              | ns                  | ns                 | 0.000565566       |
| cg02665650  | ANKS1A             | ns                  | ns                 | 0.000420543       |
| cg02683114  | C2orf84            | ns                  | ns                 | 3.28E-05          |
| cg02915422  |                   | ns                  | ns                 | 0.000993538       |
| cg03119308  | RBM28              | 0.000122845         | ns                 | ns                |
| cg03404566  | ALOX12             | ns                  | ns                 | 9.44E-05          |
| cg03407747  | ALOX12             | ns                  | ns                 | 0.000320776       |
| cg03452174  | RAB34              | ns                  | ns                 | 0.000820466       |
| cg03456213  | C9orf3             | ns                  | ns                 | 0.000620827       |
| cg03760483  | ALOX12             | ns                  | ns                 | 0.000249903       |
| cg03762994  | ALOX12             | ns                  | ns                 | 0.000338148       |
| cg03782157  |                   | ns                  | ns                 | 0.000566959       |
| cg03787864  | CYBA                | ns                  | ns                 | 0.000360395       |
| cg03955537  | TBCD               | ns                  | ns                 | 0.000449056       |
| cg03957885  |                   | ns                  | ns                 | 0.000500821       |
| cg04034767  | GRASP              | ns                  | ns                 | 0.000526517       |
| cg04178858  | RAPGEPFL1          | ns                  | ns                 | 0.000378136       |
| cg04194674  | SRCIN1             | ns                  | ns                 | 0.000665658       |
| cg04332818  | FGF2               | ns                  | ns                 | 0.000648814       |
| cg04555220  | SEMA5A             | ns                  | ns                 | 0.00094353        |
| cg04621728  |                   | ns                  | ns                 | 0.000680098       |
| cg04797170  |                   | ns                  | ns                 | 0.000729496       |
| cg05209996  |                   | ns                  | ns                 | 0.000724896       |
| cg05897210  | DTHD1              | ns                  | ns                 | 0.000252462       |
| cg05950572  | SPON1              | ns                  | ns                 | 0.000546993       |
| cg06085985  | EFCAB4A            | ns                  | ns                 | 0.000230613       |
| cg06590173  | TPM4               | ns                  | ns                 | 0.000778707       |
| cg06607764  | CYTH1              | ns                  | ns                 | 0.000254746       |
| cg06749789  | THAP4              | ns                  | ns                 | 0.000864909       |
| cg06763054  | MTMR7              | ns                  | ns                 | 0.000353509       |
| cg06795971  | TET2               | ns                  | ns                 | 0.000140266       |
| cg06835156  | C14orf70           | 0.000524942         | ns                 | 7.67E-05          |
| cg06945399  | LRRC4; SND1        | ns                  | ns                 | 0.000590044       |
| cg07016556  | BAHCC1             | ns                  | ns                 | 0.000661791       |
| cg07235805  | PARD6G             | ns                  | ns                 | 0.000689192       |
| cg07251099  | CD200              | ns                  | ns                 | 0.000689192       |
Table III. Continued.

| Target ID    | Gene name                     | P-value (GT vs. PT) | P-value (GT vs. N) | P-value (PT vs. N) |
|--------------|-------------------------------|--------------------|--------------------|-------------------|
| cg07522516   | ZAR1                          | ns                 | ns                 | 0.0006922555      |
| cg07834955   | SFRP5                         | ns                 | ns                 | 0.000372927       |
| cg07871590   | LRRC4; SND1                   | ns                 | ns                 | 0.000127567       |
| cg07924363   | MGC16121; MIR424; MIR503      | ns                 | 0.000320255        | ns                |
| cg08194377   | ANKS1A                        | ns                 | ns                 | 0.000793165       |
| cg08248285   | CFL2                          | ns                 | ns                 | 0.000346449       |
| cg08298946   | ns                            | ns                 | 0.000455024        | ns                |
| cg08330950   | ns                            | ns                 | 0.000195062        | ns                |
| cg08421126   | HAAO                          | ns                 | ns                 | 0.000388824       |
| cg08572315   | ns                            | ns                 | ns                 | 0.000667361       |
| cg08617833   | SMARCA1                       | ns                 | ns                 | 0.000373883       |
| cg09088834   | NINL                          | ns                 | ns                 | 0.000442225       |
| cg09246479   | C22orf45; UPB1                | ns                 | ns                 | 0.00010158        |
| cg09456782   | TMCO3; DCUN1D2                | ns                 | ns                 | 0.000792285       |
| cg09480054   | HAAO                          | ns                 | ns                 | 0.000295903       |
| cg09580336   | ATP1A1                        | ns                 | ns                 | 0.000440859       |
| cg09581551   | SOBP                          | ns                 | ns                 | 0.000280079       |
| cg09667289   | FMN1                          | ns                 | ns                 | 0.000712725       |
| cg09737314   | ALOX12                        | ns                 | ns                 | 0.000653337       |
| cg09920557   | ACE                           | ns                 | ns                 | 0.000673976       |
| cg09963123   | FLJ13197; KLF3                | ns                 | ns                 | 0.000654359       |
| cg10445911   | SOSTDC1                       | ns                 | ns                 | 0.000375888       |
| cg11417025   | ns                            | ns                 | ns                 | 0.000826709       |
| cg11942956   | EYA4                          | ns                 | ns                 | 0.00073108        |
| cg12177793   | NFATC4                        | ns                 | ns                 | 0.000965995       |
| cg12262378   | ALOX12                        | ns                 | ns                 | 0.000115607       |
| cg12451530   | LOC100302652; GPR75           | ns                 | ns                 | 0.000188564       |
| cg12828075   | INSC                          | ns                 | ns                 | 0.000784835       |
| cg13616314   | HS3ST3A1                      | ns                 | ns                 | 2.38E-05          |
| cg13801416   | AKR1B1                        | ns                 | ns                 | 0.000474669       |
| cg13857811   | SLC7A3                        | ns                 | ns                 | 0.000228168       |
| cg14032732   | ECHDC3                        | ns                 | ns                 | 0.000256212       |
| cg14243778   | CNTN1                         | ns                 | ns                 | 0.00077315        |
| cg14254720   | LRRC8C                        | ns                 | ns                 | 0.000920384       |
| cg14287235   | ADcy4                         | ns                 | ns                 | 0.000228476       |
| cg14482902   | SRCIN1                        | ns                 | ns                 | 0.000344968       |
| cg14500300   | ns                            | ns                 | ns                 | 8.80E-05          |
| cg14603620   | RAPGEFL1                      | ns                 | ns                 | 7.94E-05          |
| cg14663984   | AGRN                          | ns                 | ns                 | 0.000843468       |
| cg14792081   | ns                            | ns                 | ns                 | 0.000344126       |
| cg15115171   | ns                            | ns                 | ns                 | 0.000503309       |
| cg15673034   | DLGAP1                        | ns                 | ns                 | 0.000846318       |
| cg15826437   | RAPGEFL1                      | ns                 | ns                 | 0.000299995       |
| cg15998779   | ns                            | ns                 | ns                 | 0.000211956       |
| cg16450577   | TBCD                          | ns                 | ns                 | 0.000368573       |
| cg16859884   | ns                            | ns                 | ns                 | 0.000247308       |
| cg16968985   | SEZ6                          | ns                 | ns                 | 0.000382576       |
| cg17011709   | CYP26C1                        | ns                 | ns                 | 0.000901702       |
| cg17131553   | TRPS1                         | ns                 | ns                 | 0.000583708       |
| cg17165580   | CRABP2                        | ns                 | ns                 | 0.000197886       |
| cg17479501   | TBCD                          | ns                 | ns                 | 0.000197189       |
| cg17496661   | ns                            | 0.000436474        | 0.000197741        |
| cg17624073   | BAHCC1                        | ns                 | ns                 | 0.000526316       |
| Target ID      | Gene name       | P-value (GT vs. PT) | P-value (GT vs. N) | P-value (PT vs. N) |
|---------------|----------------|---------------------|-------------------|-------------------|
| cg17729667    | NINL            | ns                  | ns                | 0.000569462       |
| cg18344652    | CNN3            | ns                  | ns                | 0.000452391       |
| cg19372602    |                |                     |                   | 0.000864447       |
| cg19467964    | TBCD            | ns                  | ns                | 0.000196505       |
| cg19499884    | LZTS2           | ns                  | ns                | 0.000537829       |
| cg19929126    | TRIL            |                     |                   | 0.000632594       |
| cg20132775    | TRPC1           |                     |                   | 0.000197515       |
| cg20145692    | COL9A2          |                     |                   | 0.000190537       |
| cg20276377    | C3orf26; FILIP1L; MIR548G | ns | ns | 6.22E-05 |
| cg20383155    | NEU1; SLC44A4   |                     |                   | 0.000632549       |
| cg20801007    | EFCAB4A         | ns                  | ns                | 0.000259905       |
| cg20987431    | ZHX1            |                     |                   | 0.00053928        |
| cg21079003    | RGMA            |                     |                   | 0.000411886       |
| cg21116447    | NEU1; SLC44A4   | ns                  | ns                | 0.000990119       |
| cg21543859    | RUNX2           |                     |                   | 0.000760409       |
| cg21849932    | LIME1           | ns                  | ns                | 0.000537283       |
| cg21944491    | LTBTP4          |                     |                   | 0.000572287       |
| cg22074576    | OSBPL5          |                     |                   | 0.00073274        |
| cg22092811    | C3orf26; FILIP1L; MIR548G | ns | ns | 4.30E-05 |
| cg22413388    | WNT7B           |                     |                   | 0.000992683       |
| cg22534145    | SSTR4           |                     |                   | 0.000156886       |
| cg22675801    | TRIL            |                     |                   | 0.000451146       |
| cg22753340    | NEU1; SLC44A4   | ns                  | ns                | 0.000874186       |
| cg22773555    | EFCAB4A         | ns                  | ns                | 0.00025263        |
| cg22773661    | ZAR1            |                     |                   | 0.00033279        |
| cg22871668    | EYA4            |                     |                   | 0.000392704       |
| cg22878441    |                |                     |                   | 0.000393322       |
| cg23083315    | FIX1            |                     |                   | 0.000288759       |
| cg23142799    | SHISA2          |                     |                   | 0.000157373       |
| cg23396786    | SFXN5           |                     |                   | 0.000434986       |
| cg23425970    | HS6ST1          |                     |                   | 0.00016049        |
| cg23563927    | C10orf93        |                     |                   | 0.000585909       |
| cg23684878    |                |                     |                   | 0.000735566       |
| cg23926436    |                |                     |                   | 0.00082097        |
| cg24251193    | CRABP2          |                     |                   | 0.000141885       |
| cg24331301    | CDH23           |                     |                   | 0.000549748       |
| cg24878115    | SSBBP4          |                     |                   | 0.000354342       |
| cg24902339    | CASC2           |                     |                   | 0.000256574       |
| cg25027125    | CFL2            |                     |                   | 0.000978881       |
| cg25117523    | CYTH1           |                     |                   | 0.000297582       |
| cg25387565    | NEU1            |                     |                   | 0.000708206       |
| cg25563256    | FGF11           |                     |                   | 0.000933724       |
| cg25813864    | RAPGEFL1        |                     |                   | 0.000174816       |
| cg25834415    | KIF1A           |                     |                   | 0.000894051       |
| cg26009486    | NFATC4          |                     |                   | 0.000293111       |
| cg26360792    | HAAO            |                     |                   | 0.000297095       |
| cg26558799    | TBCD            |                     |                   | 0.000570916       |
| cg26607748    | TPM2            |                     |                   | 0.000773141       |
| cg26846076    | CYTSB           | 0.000457469         | ns                | ns                |
| cg27191312    |                |                     |                   | 0.00012339        |
| cg27299406    | HAAO            |                     |                   | 0.000380895       |
| cg27347290    | NEU1; SLC44A4   | ns                  | ns                | 0.000429935       |
| cg27573591    | SND1; LRRC4     | ns                  | ns                | 0.000183694       |
| rs10033147    |                | 0.00000393          | ns                | ns                |

GT, genistein-treated tumor; PT, placebo-treated tumor; N, normal; NS, not significant.
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by microarrays using qPCR, and observed that microarray data were correlated with qPCR results (Fig. 2). The increase in the qPCR expression levels of NOTCH3 and JAG1 genes in genistein-treated tumors compared to placebo-treated tumors was statistically significant by Mann-Whitney U test.

**Enrichment analysis.** We performed gene enrichment analysis on the 628 nominally significant probes that were differentially expressed between genistein and placebo samples (Table V) using Ingenuity Pathway Analysis (62) and the DAVID Knowledgebase (63). P-value indicates hypergeometric distribution P-values of overlap for gene sets and functional categories. FDR indicates false discovery rate corrected P-values of overlap. Activation z-score is an indication of the consistency of up and downregulated members of a gene set such as a biological function (top table) or targets of an upstream regulator (middle table). Activation z-scores >2 or < -2 are statistically significant for consistency of activation or inhibition. Molecules indicate the number of molecules in the set of 628 analyzed probes that overlap with a given category. Mechanistic network indicates the total number of target genes of an upstream regulator, and the number of overlapping genes is indicated in parentheses. We observed enrichment for terms associated with angiogenesis, apoptosis, epithelial to mesenchymal transition, tumor progression and PDGF binding. Analysis of potential upstream regulators by IPA analysis suggested that PTEN and PDGF were activated, while MYC, β-estradiol, glucocorticoid receptor NR3C1 and interferon-γ were repressed in response to genistein treatment.

**Discussion**

To the best of our knowledge, the present study is the first highlighting the effects of genistein on global changes in DNA methylation status is correlated with gene expression in NEU1 and ADCY4. qPCR, quantitative PCR.
methyltransferase inhibitor, thereby causing the demethylation of CpG islands in the promoters of genes. For example, genistein has been shown to reactivate the hypermethylated-silenced tumor suppressor genes, including \(p16^{INK4a}\), retinoic acid receptor \(\beta\) (\(RAR\beta\)) and \(O_6\)-methylguanine methyltransferase (\(MGMT\)), in prostate and esophageal cancer cells (46). Moreover, genistein has been implicated in demethylation of \(WNT5a\) promoter in colon cancer cells (64). One of the genes shown to be demethylated by genistein in the present study is \(ADCY4\), which is a member of the family of adenylate cyclases, the membrane-bound enzymes that catalyze formation of the secondary messenger cyclic adenosine monophosphate (cAMP) (65). Consistent with our finding, it has been recently shown that \(ADCY4\) is a DNA methylation marker representing early epigenetic events in prostate tumorigenesis, supporting our hypothesis that genistein may reverse the pattern of DNA methylation in \(ADCY4\) in prostate cancer (66). The other gene that was modulated by genistein intervention in the present study was \(NEU1\), which is a lysosomal sialidase involved in glycoconjugate catabolism and cellular signaling, including immune responses and elastin

Table V. Enrichment analysis of 628 nominally significant probes differentially expressed between genistein and placebo groups.

| Analysis | P-value | Activation z-score | No. of molecules | Function |
|----------|---------|--------------------|------------------|----------|
| IPA      | 5.92E-08 | 0.773              | 18               | Progression of tumor |
| IPA      | 4.88E-07 | 1.01               | 355              | Abdominal neoplasm |
| IPA      | 1.09E-06 | 1.927              | 28               | Differentiation of tumor cell lines |
| IPA      | 1.34E-06 | -1.017             | 19               | Epithelial-mesenchymal transition |
| IPA      | 7.46E-06 | 2.412              | 22               | Neuroendocrine tumor |
| IPA      | 7.98E-05 | 2.054              | 28               | Necrosis of tumor |

| Analysis | P-value of overlap | Activation z-score | Mechanistic network | Upstream regulator |
|----------|-------------------|--------------------|---------------------|--------------------|
| IPA      | 3.85E-08          | -0.692             | 184 (16)            | NR3C1              |
| IPA      | 1.21E-07          | 1.681              | 112 (9)             | PDGFB              |
| IPA      | 2.71E-07          | -1.385             | 167 (15)            | \(\beta\)-estradiol |
| IPA      | 2.15E-06          | -0.832             | 144 (13)            | IFNG               |
| IPA      | 2.17E-06          | 1.608              | 141 (16)            | PTEN               |
| IPA      | 4.59E-06          | -2.995             | 133 (13)            | MYC                |

| Analysis | FDR | Activation z-score | No. of molecules | Term |
|----------|-----|--------------------|------------------|------|
| DAVID    | 7.90E-04 | NA                | 17               | GO:0005840 ribosome |
| DAVID    | 1.19E-02 | NA                | 34               | mitochondrion     |
| DAVID    | 2.00E-02 | NA                | 16               | GO:0001568 blood vessel development |
| DAVID    | 1.77E-02 | NA                | 10               | GO:0019838 growth factor binding |
| DAVID    | 3.52E-02 | NA                | 7                | GO:0008629 induction of apoptosis by intracellular signals |
| DAVID    | 3.16E-02 | NA                | 4                | GO:0048407 platelet-derived growth factor binding |

It is of interest to note that DNA methylation status was inversely correlated with gene expression for the \(NEU1\) and \(ADCY4\) genes, which had decreased methylation, and increased mRNA expression in the genistein group in comparison with placebo group. Our finding showing the potential of genistein for DNA demethylation is consistent with the previously reported data that suggest genistein acts as a DNMT inhibitor, thereby causing the demethylation of CpG islands in the promoters of genes. For example, genistein has been shown to reactivate the hypermethylated-silenced tumor suppressor genes, including \(p16^{INK4a}\), retinoic acid receptor \(\beta\) (\(RAR\beta\)) and \(O_6\)-methylguanine methyltransferase (\(MGMT\)), in prostate and esophageal cancer cells (46). Moreover, genistein has been implicated in demethylation of \(WNT5a\) promoter in colon cancer cells (64). One of the genes shown to be demethylated by genistein in the present study is \(ADCY4\), which is a member of the family of adenylate cyclases, the membrane-bound enzymes that catalyze formation of the secondary messenger cyclic adenosine monophosphate (cAMP) (65). Consistent with our finding, it has been recently shown that \(ADCY4\) is a DNA methylation marker representing early epigenetic events in prostate tumorigenesis, supporting our hypothesis that genistein may reverse the pattern of DNA methylation in \(ADCY4\) in prostate cancer (66). The other gene that was modulated by genistein intervention in the present study was \(NEU1\), which is a lysosomal sialidase involved in glycoconjugate catabolism and cellular signaling, including immune responses and elastin.
receptor-mediated signal transduction (67). In fact, NEU1 is critical for desialylation of integrin β4 and inhibition of FAK, leading to suppression of liver metastases in colon cancer (68). Kato et al (69) has reported that NEU1 overexpression resulted in suppression of lung metastasis in melanoma. In addition, suppression of NEU1 by miR-125b has been shown to promote migration, invasion and metastasis in gastric cancers (70). However, NEU1 can also have pro-metastatic effects in pancreatic and ovarian cancers (71), and thus it is not entirely clear what the overall impact of increased NEU1 levels might be in prostate cancer. Therefore, it is important to examine the NEU1 expression changes at the protein level, and molecular and cellular studies are required to assess the functional consequences of changes induced by NEU1 upregulation in prostate cancer cells.

Among the differentially expressed genes that were validated by qPCR, only the expression of NOTCH3 and JAG1 mRNAs were significantly higher in the genistein group compared to the placebo group by qPCR. Based on our findings at mRNA level without any confirmation at the protein or functional level, it would be speculative to suggest that Notch signaling may play a role in the mechanism of action of genistein on prostate cancer. NOTCH3 is important for TGFβ-induced EMT in prostate cancer (72), and is induced by hypoxia and contributes to prostate cancer progression (73). The Notch ligand JAG1 is also associated with more aggressive prostate cancer (74,75), EMT and angiogenesis (76). However, a tumor suppressive role of Notch signaling has also been reported in hypoxia-induced neuroendocrine differentiation of prostate cancer cells as well as in other cancer types including bladder cancer, hematological malignancies, glioma, thyroid carcinoma and lung cancer (77-82), indicating the possibility that increased NOTCH3/JAG1 expression by genistein treatment may improve outcomes through its tumor suppressor function. Our data suggest that further studies to delineate the effect of genistein on the Notch signaling pathway in prostate cancer may be warranted.

Enrichment analyses of mRNA changes induced by genistein indicated that subtle changes in gene expression observed between genistein and placebo samples are consistent with many previously reported effects of genistein on critical tumor pathways including PTEN, PDGF, MYC, β-estradiol, glucocorticoid receptor and interferon-γ (41,83-89). Genistein appeared to promote PTEN activity and inhibit MYC activity, consistent with its potential utility in improving outcomes in prostate cancer.

In summary, our results indicate that genistein intervention induces modulation of several genes, including NOTCH3, JAG1, ADCY4 and NEU1, suggesting that these genes may have the potential to be novel molecular targets of genistein in prostate cancer. These genes are involved in many critical biological processes including cell cycle, angiogenesis, cellular immune response and intracellular signal transduction, providing additional insight into the multiple molecular pathways involved in prostate tumorigenesis. However, further mechanistic studies are required to investigate the effects of genistein on the regulation of the expression of these genes at the protein level and cellular functions. These findings may then contribute towards designing novel strategies for prevention and treatment of prostate cancer. One caveat of gene expression profiling studies is the incapability of identification of mechanisms of action that are modulated at post-transcriptional level, suggesting the possibility that genistein may alter additional cellular processes. Another point that needs to be made is timing and duration of exposure to genistein. Case control studies have demonstrated that high consumption of soy early in life (during childhood and/or adolescence) is associated with 25-60% reductions in breast cancer risk (90,91). Similarly, high soy intake at puberty, the period during which prostate undergoes androgen-induced growth, might be more effective in prevention of prostate cancer. A limitation of the present study is the small number of patient samples. Further large randomized controlled clinical trials would provide more definitive results of the effects of genistein on patient prostate tissues.

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