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.

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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, Aker.

Genome-wide methylation profiling. Total DNA was isolated from frozen prostate tissues using DNeasy 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 I. 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

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

Primer name	Primer sequence (5'→3')
CKS2-FP	TTAGTCTCCGGCGAGTTGTTG
CKS2-RP	CATAACATGCCGGTACTCGT
JAG1-FP	AGTCGTGCATGCTCCAATCG
JAG1-RP	CCCCACACACCTTGGCTC
NOTCH3-FP	GATGTGGACGAGTGTGCTGG
NOTCH3-RP	CAGGCATGGGTTGGGGTC
MMP26-FP	GGACTTTGTTGAGGGCTATTTCCA
MMP26-RP	GGAGGTGTCGGACCCATCAG
HIF1A-FP	CACCACAGGACAGTACAGGAT
HIF1A-RP	CGTGCTGAATAATACCACTCACA
CDK6-FP	GCTGACCAGCAGTACGAA TG
CDK6-RP	GCACACATCAAACAACCTGACC
CD24-FP	CGCGGACTTTTCTTTTGGGG
CD24-RP	ACTGGAATAAATCTGCGTGGGT
AMACR-FP	CCGTTCTGTGCTATGGTCCTG
AMACR-RP	AGCCTTGGATTTTCCCGCTG
MYC-FP	CCTACCCTCTCAACGACAGC
MYC-RP	TTGTTCCTCCTCAGAGTCGC
SPP1-FP	CAAACGCCGACCAAGGAAAA
SPP1-RP	GGCCACAGCATCTGGGTATT
NEU1-FP	CGCAGCTATGATGCCTGTGA
NEU1-RP	GGTCAGGTTCACTCGGAACTC
ADCY4-FP	CCTGGGACCAGGTGTCCTAT
ADCY4-RP	CAAGATACAGCCCGAGGACC
β-actin-FP	CACAGAGCCTCGCCTTTGCC
β-actin-RP	TGACCCATGCCCACCATCAC

qPCR analysis.

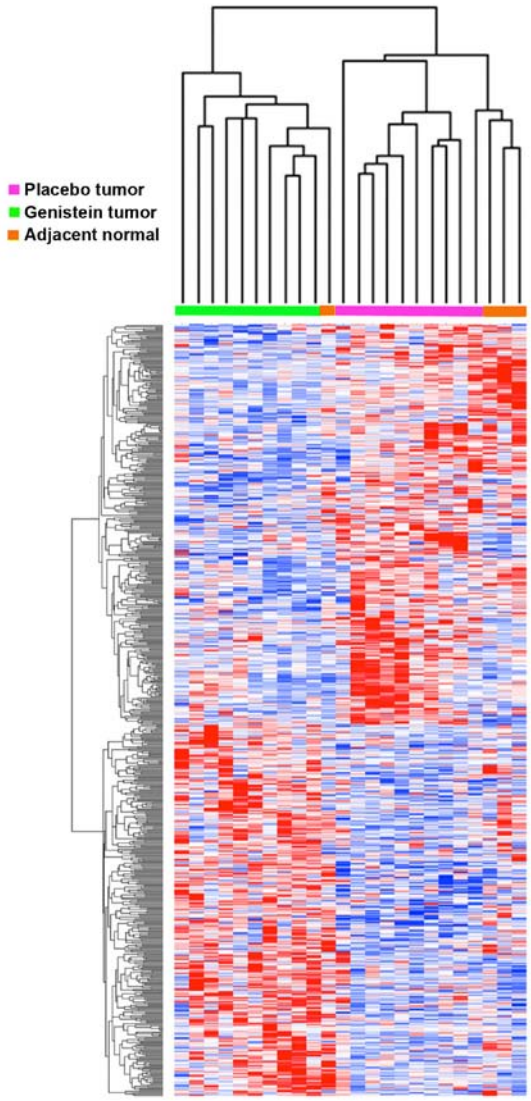

Figure 1. Whole genome expression profiling of placebo- or genistein-treated tumor samples. Hierarchical clustering of changes in gene expression for those genes with a nominal P<0.05 between genistein-tumor samples and placebo-tumor samples.

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 β-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 II. Clinical data for the 20 patients analyzed in the present study.

Treatment	Patient ID	Gleason	Gleason Sum	Stage	Age	PSA
Genistein (n=10)	1	3+4	7	2	68	12.0
	7	3+4	7	2	68	10.9
	8	3+3	6	2	59	6.2
	10	3+4	7	3a	61	5.1
	13	3+3	6	2	58	7.6
	14	4+3	7	3a	64	8.5
	17	4+4	8	2	61	6.1
	18	3+3	6	2	57	6.0
	19	3+3	6	2	63	7.8
	20	3+4	7	2	68	7.9
Average (SD)			6.7 (0.7)		62.7 (4.2)	7.8 (2.2)
Placebo (n=10)	24	3+3	6	2	61	6.4
	25	3+3	6	2	57	4.2
	26	3+4	7	3a	68	9.9
	27	4+4	8	2	69	9.2
	30	4+3	7	2	55	5.1
	33	3+3	6	2	56	6.4
	34	3+3	6	2	63	7.6
	35	3+4	7	3a	60	5.7
	38	3+4	7	2	62	9.9
	39	3+3	6	2	66	7.0
Average (SD)			6.6 (0.7)		61.7 (4.9)	7.1 (2.0)

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). Hierarchial 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*, *CKS2*, *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	RAPGEFL1	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	0.00015216
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	EPS8L2	ns	ns	0.000563556
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	RAPGEFL1	ns	ns	0.000378136
cg04194674	SRCIN1	ns	ns	0.000665658
cg04332818	FGF2	ns	ns	0.000648814
cg04555220	SEMA5A	ns	ns	0.000994353
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	ns	0.000524942	ns
cg06945399	LRRC4; SND1	ns	ns	7.67E-05
cg07016556	BAHCC1	ns	ns	0.000590044
cg07235805	PARD6G	ns	ns	0.000661791
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	0.000692555
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
cg08330950		ns	ns	0.000195062
cg08421126	HAAO	ns	ns	0.000388422
cg08572315		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	0.000792785
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	0.000673337
cg09920557	ACE	ns	ns	0.000673976
cg09963123	FLJ13197; KLF3	ns	ns	0.000654359
cg10445911		ns	ns	0.00061326
cg11417025	SOSTDC1	ns	ns	0.000375888
cg11832404		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	8.80E-05
cg14603620	RAPGEFL1	ns	ns	7.94E-05
cg14663984	AGRN	ns	ns	0.000843468
cg14792081		ns	ns	0.000344126
cg15115171		ns	ns	0.000503109
cg15673034	DLGAP1	ns	ns	0.000846318
cg15826437	RAPGEFL1	ns	ns	0.00029995
cg15998779		ns	ns	0.000211956
cg16450577	TBCD	ns	ns	0.000368573
cg16859884		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	0.000459741
cg17624073	BAHCC1	ns	ns	0.000526316

Table III. Continued.

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		ns	ns	0.000864447
cg19467964	TBCD	ns	ns	0.000196505
cg19499884	LZTS2	ns	ns	0.000537829
cg19929126	TRIL	ns	ns	0.000632594
cg20132775	TRPC1	ns	ns	0.000197515
cg20145692	COL9A2	ns	ns	0.000190537
cg20276377	C3orf26; FILIP1L; MIR548G	ns	ns	6.22E-05
cg20383155	NEU1; SLC44A4	ns	ns	0.000632549
cg20801007	EFCAB4A	ns	ns	0.000259905
cg20987431	ZHX1	ns	ns	0.00053928
cg21079003	RGMA	ns	ns	0.000411886
cg21116447	NEU1; SLC44A4	ns	ns	0.000990119
cg21543859	RUNX2	ns	ns	0.000760409
cg21849932	LIME1	ns	ns	0.000537283
cg21944491	LTBP4	ns	ns	0.000572287
cg22074576	OSBPL5	ns	ns	0.00073274
cg22092811	C3orf26; FILIP1L; MIR548G	ns	ns	4.30E-05
cg22413388	WNT7B	ns	ns	0.000992683
cg22534145	SSTR4	ns	ns	0.000156886
cg22675801	TRIL	ns	ns	0.000451146
cg22753340	NEU1; SLC44A4	ns	ns	0.000874186
cg22773555	EFCAB4A	ns	ns	0.00025263
cg22773661	ZAR1	ns	ns	0.00033279
cg22871668	EYA4	ns	ns	0.000392704
cg22878441		ns	ns	0.000393322
cg23083315	FJX1	ns	ns	0.000288759
cg23142799	SHISA2	ns	ns	0.000157373
cg23396786	SFXN5	ns	ns	0.000434986
cg23425970	HS6ST1	ns	ns	0.00016049
cg23563927	C10orf93	ns	ns	0.000585909
cg23684878		ns	ns	0.000735566
cg23926436		ns	ns	0.00082097
cg24251193	CRABP2	ns	ns	0.000141885
cg24331301	CDH23	ns	ns	0.000549748
cg24878115	SSBP4	ns	ns	0.000354342
cg24902339	CASC2	ns	ns	0.000256574
cg25027125	CFL2	ns	ns	0.000978881
cg25117523	CYTH1	ns	ns	0.000297582
cg25387565	NEU1	ns	ns	0.000708206
cg25563256	FGF11	ns	ns	0.000933724
cg25813864	RAPGEFL1	ns	ns	0.000174816
cg25834415	KIF1A	ns	ns	0.000894051
cg26009486	NFATC4	ns	ns	0.000293111
cg26360792	HAAO	ns	ns	0.000297095
cg26558799	TBCD	ns	ns	0.000570916
cg26607748	TPM2	ns	ns	0.000773141
cg26846076	CYTSB	0.000457469	ns	ns
cg27191312		ns	ns	0.00012339
cg27299406	HAAO	ns	ns	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.

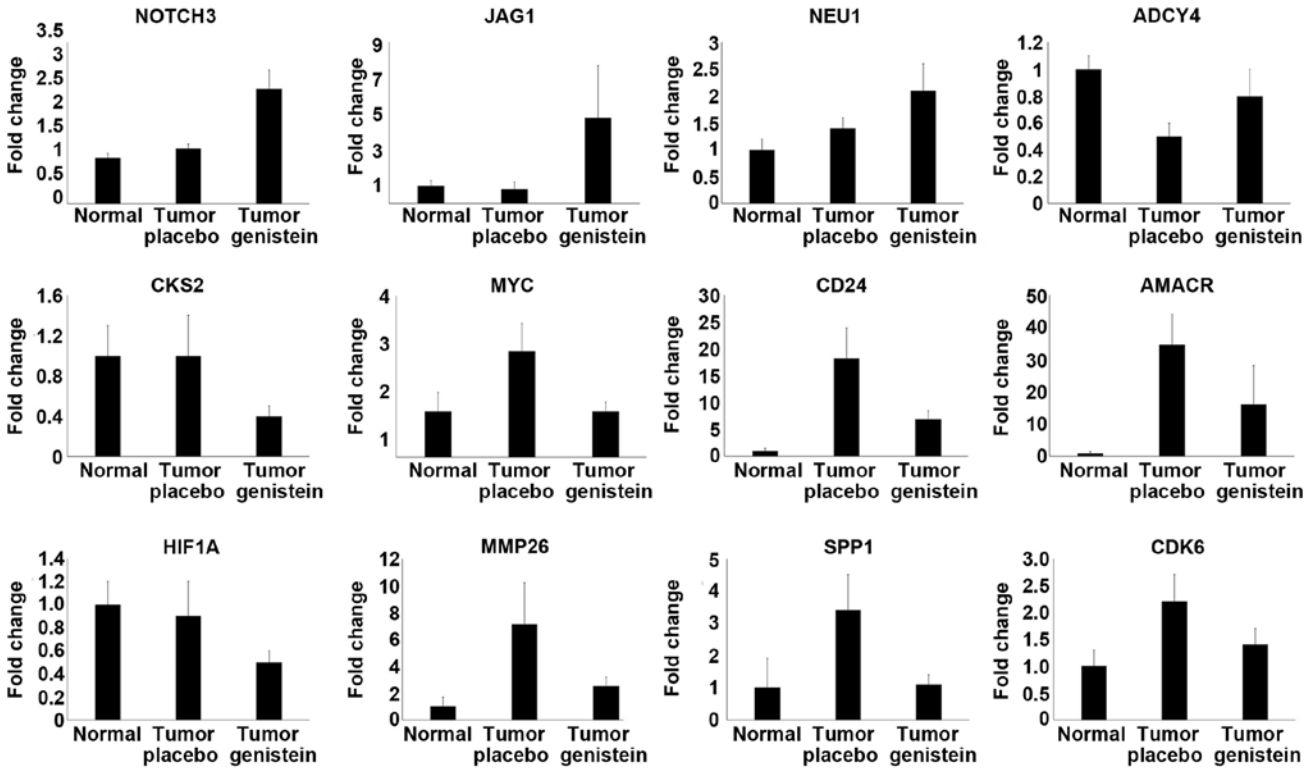

Figure 2. qPCR analysis of expression levels of 12 genes in placebo and genistein groups. Expression changes of the genes selected from the microarray data were validated using qPCR. The data are presented as fold-changes relative to the control samples. qPCR, quantitative PCR.

Table IV. Genes with differential gene expression analyzed by qPCR.

	Gene expression fold-change	
Gene symbol	Microarray	qPCR
CKS2	-2.02	-2.50
NOTCH3	1.72	2.08
HIF1A	-1.63	-1.80
CDK6	-2.87	-1.57
JAG1	1.91	6.00
NEU1[a]	1.77	1.50
ADCY4[a]	1.67	1.60
MYC	-1.57	-2.30
CD24	-2.02	-2.64
AMACR	-1.95	-2.14
MMPP26	-2.78	-2.84
SPP1	-2.36	-3.09

Fold changes are genistein-tumor/placebo-tumor. [a]DNA methylation status is correlated with gene expression in *NEU1* and *ADCY4*. qPCR, quantitative PCR.

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 were 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

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)	β-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

methylation and gene expression in patients from a clinical trial of genistein in prostate cancer. Integrative analysis of whole genome methylation and expression profiling identified a number of candidate differentially methylated sites and expressed sites between placebo and genistein groups. However, the differences between placebo and genistein groups were not statistically significant after correction for multiple hypothesis testing, possibly due to the small number of the cases in this study. Although the genistein-induced alterations are not significant, these results may help to elucidate the molecular mechanisms underlying the activities of genistein in prostate cancer. Genome-wide DNA methylation arrays showed that a number of genes, including *RBM28* and *CYTSB*, appeared to be demethylated in the genistein-treated tumor samples compared to the samples in the placebo group. However, we did not observe any alterations in the expression levels of these genes. Among the differentially expressed genes identified by microarray analysis were *CKS2*, *NOTCH3*, *HIF1A*, *CDK6*, *JAG1*, *NEU1*, *ADCY4*, *MYC*, *CD24*, *AMACR*, *MMP26* and *SPP1*. Microarray data were confirmed by qPCR analysis of these genes. Other genes with nominal significance by microarray but not tested by qPCR included *ZNF639*, *CRIM1*, *PGC* and *USP54* (available upon request).

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 *p16INK4a*, retinoic acid receptor β (*RARβ*) 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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