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Original Article

Genistein-Induced Proteome Changes in the Human Endometrial Carcinoma Cell Line, Ishikawa

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Abstract

Epidemiological studies have shown that Asian populations display a lower incidence of hormone-dependant cancers, cardiovascular disease, osteoporosis, and menopausal ailments compared to Western societies. Available data support the proposal that lower incidence is associated with the high dietary consumption of isoflavones, such as genistein. This study used two-dimensional electrophoresis to characterize the effect of genistein on the proteome of an endometrial tumor cell model, namely the Ishikawa cell line. Proteome maps displaying approx 1800 proteins were obtained from cells treated with vehicle or genistein at physiologically attainable concentrations of 0.5, 5, or 50 µ*M* or supra-physiological concentration, 500 µ*M*. The effects of genistein on protein expression were characterized using image analysis software. A total 65 protein spots displayed a significant decrease in expression and 32 proteins displayed a significant increase in expression. Of these protein spots, 29 were randomly selected for characterization by matrix assisted laser desorption/ionization tandem

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mass spectrometry, yielding 18 different proteins. This type of analysis enabled the characterization of a wide range of cellular proteins and allowed for the identification of functional and biochemical pathways that may be regulated or affected by genistein, including cellular transcription, cell proliferation, stress response, or modulation of oncogenic pathways.

Key Words: Genistein; endometrial cancer; proteomics; phytoestrogen; tumor suppression.

Introduction

Epidemiological studies have shown that Asian populations display a lower incidence of hormone-dependant cancers, cardiovascular disease, osteoporosis, and menopausal ailments compared to Western societies ***(1,2)***. The difference in incidence has been attributed to environmental factors, predominantly diet. This hypothesis is based upon data obtained in studies which report that people from Asia that migrate to a Western nation and adopt a Western diet display similar incidence of disease as their adoptive country by the second generation, suggesting that factors other than genetic factors are influential ***(3)***.

Western societies typically consume diets that are relatively high in animal fat and low in fiber, as opposed to Asian societies, which consume plant-based diets, rich in phytoestrogens. Phytoestrogens are plant-derived compound with structural similarity to human estrogenic steroids. Generally, Asian populations consume 20–50 mg of isoflavones in the daily diet. In contrast, Western populations ingest negligible amounts (<1 mg isoflavone) per day ***(4)***.

Genistein, the most prominent isoflavone in soybeans, has been extensively studied as a chemopreventive or therapeutic agent in several forms of cancer ***(5)***. Over the past decade, many studies have focused on elucidating which, if any, cellular mechanisms are regulated by genistein that may lead to protection from cancer. In vitro studies have demonstrated that genistein displays biphasic effects on cancer cell growth, stimulating growth at low concentrations (<10 μ*M*) and inhibiting growth at higher concentrations (>10 μ*M*) ***(6)***. It binds to both estrogen receptor α and β and elicits a weak estrogenic response. Furthermore, genistein acts as a protein tyrosine kinase inhibitor, and is able to block the cell cycle in the G2/M transition, inhibit Akt kinase, topiosomerase II, and cAMP-phosphodiesterase-4 enzymes ***(7–12)***.

Studies to date have typically focused on specific candidate pathways previously associated with cancer. By focusing on the regulation of such pathways, other putative effects of genistein on cellular function remain to be elucidated. Changes to the cellular proteome elucidated by phytoestrogens have also been reported, however these studies are, to date, limited ***(13–16)***. Recently, several studies have reported the use of two-dimensional electrophoresis (2D-PAGE) technology to identify proteome changed elucidated by genistein ***(13,15–17)***.

In this study, 2D-PAGE technology was used to characterise changes in the proteome elicited in an endometrial carcinoma cell line, Ishikawa, treated with previously reported physiologically attainable genistein concentrations, 0.5, 5, and 50 μ*M* ***(18–22)*** and the supraphysiological concentration, 500 μ*M* compared to vehicle treated cells. This type of analysis was employed as it enabled the characterization of a wider range of cellular proteins and possible biochemical pathways that may be regulated or affected by genistein. It is a powerful and widely used method for the analysis of complex protein mixtures extracted from cells ***(23–25)***.

Materials and Methods

Cells and Chemicals

The estrogen receptor α- and β-positive endometrial carcinoma cell line, Ishikawa, was kindly provided by Dr. H. Sokamoto ***(26)*** (Nihon University, Tokyo, Japan). The following reagents were purchased from Sigma (St. Louis, MO); CHAPS, α-cyano-4-hydroxycinnamic acid, genistein (4′,5,7- trihydroxyisoflavone), Hanks' balanced salt solution (Hanks), $MgCl_2$, phenylmethanesulfonyl fluoride, sodium dodecyl sulfate (SDS), thiourea, and tributylphosphine. Eagle's minimal medium without phenol red was purchased from Gibco BRL (Auckland, New Zealand). Fetal bovine serum (FBS) was purchased from CSL (Melbourne, Australia). Penicillin/streptomycin and trypsin was purchased from Trace Scientific (Melbourne, Australia). Acetonitrile, dimethyl sulphoxide (DMSO), formic acid, methanol, orthophosphoric acid, and Tris-HCl were purchased from Merck (Darmstadt, Germany). The Coomassie protein assay kit was purchased from Pierce (Rockford, IL). Coomassie R-250 and Coomassie G-250, deoxyribonuclease, glycine, and ribodeoxynuclease were purchased from ICN Pharmaceuticals (Costa Mesa, CA). Agarose M, bromophenol blue, dithiothreitol, IPG buffer 3–10 L, IPG polyacrylamide linear p*I* 3–10 strips were all purchased from GE Healthcare (Uppsala, Sweden). Modified trypsin was from Promega (Madison, WI). Gel loader tips were from Eppendorf (Hamburg, Germany). Poros R2 material was from Applied Biosystems (Framingham, MA). Broad range molecular weight markers and electrophoresis wicks and SYPRO Ruby gel stain were purchased from Bio-Rad Laboratories (Hercules, CA).

Cell Culture and Genistein Treatment

Ishikawa cells were cultured in 10 mL Eagle's minimal medium without phenol red, supplemented with 10% charcoal-treated FBS and 1% penicillin/streptomycin, in 25-cm^2 flasks until 80% confluent. Cells were washed three times using prewarmed Hanks solution, pH 7.2, and incubated for a further 24 h in 10 mL of culture medium, without charcoal-treated FBS, at 37°C in 5% CO_2 in the presence of 0.5, 5, 50, or 500 μ*M* genistein or with vehicle alone (DMSO:methanol). All treatment groups had a final concentration of 0.5% v/v DMSO: methanol. The cells were washed three times with prewarmed Hanks, and then lysed using a solution comprising 6 *M* urea, 2 *M* thiourea, 4% CHAPS, and 4.5 m*M* phenylmethanesulfonyl fluoride. Cells were incubated at room temperature for 30 min and the lysate was collected and stored at –80°C.

DNase/RNase Treatment

Protein lysates were treated with DNase and RNase (0.1 vol of 1 mg/mL DNase and 25 mg/mL RNase diluted in 50 m*M* $MgCl_2$). Samples were incubated at room temp for 30 min, and centrifuged at 16,100*g* for 20 min. Supernatant was removed and stored at –80°C.

Protein Assay

Total protein determination was performed using a Coomassie protein assay kit, following the manufacturer's instructions.

Two-Dimensional Electrophoresis

2D-PAGE, IEF in the first dimension and SDS-PAGE in the second dimension, was performed as described by Gorg et al. with minor modifications ***(27)***. Briefly, Immobiline IPG strips were actively rehydrated in 350 μL rehydration solution (150 μg protein, 8 *M* urea, 2% CHAPS, 2% IPG buffer 3–10 L, 18 m*M* dithiothreitol, bromophenol blue) at 50 V for 850 Vh using a GE Healthcare IPGPhor system. Wicks were placed under the electrodes and the IEF was performed at 500 V for 30 min, 1500 V for 1 h and 8000 V for 3 h until approx 24,000 Vh were reached. Strips were equilibrated for 15 min in SDS equilibration buffer (6 *M* Urea,

50 m*M* Tris-HCl pH 8.8, 30% glycerol, 2% SDS, 2 m*M* tributylphosphine, bromophenol blue) then soaked in SDS electrophoresis buffer (25 m*M* Tris, 192 m*M* glycine, 0.1% SDS) and placed on top of a 1-mm thick 12% homogenous polyacrylamide gels (prepared according to Hoefer Dalt manual). Molecular weight markers were placed on a filter paper and inserted next to the strip. Strips were then sealed using agarose sealing solution (100 mL SDS electrophoresis buffer, 0.5% agarose M, bromophenol blue). Gels were run using a Hoefer Dalt system (GE Healthcare) at 100 V at 10°C for 17 h in SDS electrophoresis buffer or until the dye front ran off the gel.

Protein Detection

Proteins were visualized using SYPRO Ruby fluorescent dye. Gels were stained for 3 h according to the manufacturer's instructions. Images were captured using a FX Pro Plus Imager (Bio-Rad Laboratories).

Quantification of Proteins

PD Quest, v6.2.1 (Bio-Rad Laboratories) was used for protein spot analysis. Initially, protein spots were matched automatically, and remaining proteins were manually matched as appropriate. Gels were normalized according to the total protein in the gel.

Statistical Analysis

Background values were subtracted from each gel and the spot volume in each gel was calculated. Statistical analysis was performed using PD Quest v6.2.1. Gels of the same treatment were grouped together to form replicate groups. Differences between groups (that is, genistein treatment vs control) were calculated using Mann-Whitney U-test, where $p < 0.05$ was regarded as significant.

Excising Protein Spots

Gels were stained in colloidal Coomassie (17% ammonium sulfate, 3% orthophosphoric acid, 0.1% Coomassie G-250, 34% methanol). After rocking for 4 h at room temperature, gels were placed in 30% methanol and rocked to destain until spots could be seen clearly (approx 2 h). Spots of interest were identified by eye, excised manually using a scalpel and placed in microcentrifuge tubes for further analysis.

In-Gel Digestion

In-gel digestion was performed as previously described ***(28)***. The supernatant from the digestion was either analyzed directly by matrix assisted laser desorption/ionization tandem mass spectrometry (MALDI-TOF/TOF) described in section MALDI-TOF/TOF protein identification, or an aliquot of the supernatant was desalted and concentrated using microcolumns prior to MALDI-TOF, as described next.

Desalting and Concentration of Peptide Mixtures

Desalting and concentration of the peptide mixtures prior to mass spectrometric analysis was performed in custom-made microcolumns containing 100–300 n/L of Poros R2 material (Applied Biosystems, 20 μm bead size) packed into a constricted GeLoader pipet tip as described previously ***(29,30)***. A 10-mL syringe was used to force liquid through the column by applying gentle air pressure. Peptide mixtures were dissolved in 5% formic acid, loaded onto the column, and washed with 20 μL of 5% formic acid. For analyses by MALDI TOF, peptides were eluted with 0.4 μL matrix solution (15–20 g/L of α-cyano-4-hydroxycinnamic acid in 70% acetonitrile/0.1% TFA) and deposited in very small droplets directly onto the MALDI target.

MALDI-TOF/TOF Protein Identification

MALDI tandem mass spectrometry was performed using a ABI 4700 Proteomics Analyzer (Applied Biosystems) with TOF/TOF™ optics, equipped with a Nd:YAG 200 Hz laser.

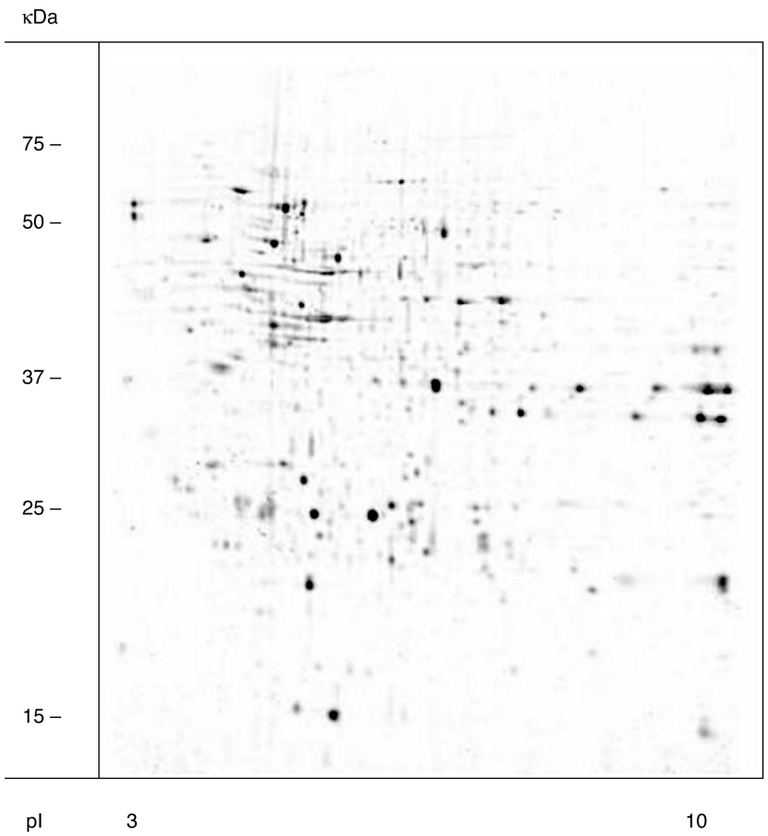

Fig. 1. Representative example of SYPRO Ruby stained 2D-PAGE gel of proteins obtained from vehicle-treated Ishikawa cells and separated using linear pH 3.0–10.0 polyacrylamide strips in the first dimension and 12% homogenous gel in the second dimension.

Spectra were obtained by accumulation of 1500–3000 consecutive laser shots. All spectra were obtained in positive reflector mode. Air was used as collision gas for collision-induced dissociation. One MS spectrum covering the mass range 700 to 4000 Da was obtained for each peptide solution. A total of seven collision-induced dissociation fragment ion spectra of the seven highest abundant peptides from each MS spectrum were obtained.

The proteins were identified based on the MALDI tandem MS data using the database search program GPS Explorer™ Workstation, employing an in-house MASCOT server (v1.8) with $p < 0.01$ (Matrix Sciences, London, UK) ***(31)***. The search was performed on both MS and MS/MS data simultaneously. The peptide mass maps and protein identifications were evaluated as previously described ***(32)***.

Results

All genistein treatments were preformed in quadruplicate. Protein content of cells following treatment with 0.5, 5, 50 μ*M* genistein or control was approx 1.7 mg/mL (SEM: 0.070), however, upon treatment with 500 μ*M* genistein the protein concentration decreased to approx 0.9 mg/mL (SEM: 0.092). Proteins (150 μg) were separated using 2D–PAGE. **Figure 1** shows a representative display of Ishikawa cell lysate focused in first dimension on an 18-cm polyacrylamide gel strip between linear

Table 1
Number of Proteins Significantly Up- or Downregulated in Ishikawa Cells[a]

Genistein concentration (μ*M*)	Upregulated (*n* = 4)	Downregulated (*n* = 4)
0.5	6	7
5	18	16
50	8	22
500	0	20

[a]Following genistein treatment with physiological concentrations, 0.5, 5, and 50 μ*M* or supra-physiological concentration, 500 μ*M* ($p < 0.05$).

p*I* of 3–10 and run on 12% SDS-PAGE gel. All gels displayed approx 1800 protein spots. The spots were separated between the pH range 3.0 and 10.0 and M_r 15 to 100 kDa, however, the majority of proteins appeared between the pH range 4.0 and 7.0 with M_r between 25 and 70 kDa.

Treatment with genistein at physiological concentrations of 0.5, 5, and 50 μ*M* resulted in significant increase in expression in 32 proteins ($p < 0.05$) and significantly decreased expression in 45 proteins ($p < 0.05$). At the supra-physiological concentration of 500 μ*M*, a total of 20 proteins displayed a significant decrease in expression ($p < 0.05$) (refer to **Table 1**).

A total of 29 gel spots that displayed significant differences in expression levels in control vs treatment were chosen at random from the various genistein treatments, gel plugs excised and proteins identified by MALDI TOF/TOF MS. To determine if PD Quest matched protein spots accurately, a matched spot was excised from gels with genistein treatments 5, 50, and 500 μ*M* and characterized, all spots were identified to be TCP1 protein. Three of the excised proteins were found to consist of two unresolved proteins. Seven spots remained unidentified, four of which did not contain a match in the database, whereas three were of very low abundance and unable to be characterized. Proteins that displayed significant differential expression ($p < 0.05$) following genistein treatment, identified using MALDI TOF/TOF MS are listed in **Table 2**. Changes in protein expression levels at all other genistein concentrations are also included in **Table 2**.

The proteins identified in all treatment concentrations are associated with cellular transcription, cell proliferation, stress response, or modulation of oncogenic pathways.

Discussion

Genistein has been widely studied over the past decade to identify putative chemopreventative properties. Various biological signal transduction pathways associated with the development of cancer, including nuclear factor (NF)-κB, AKT, and MEKK are regulated by genistein ***(8,33,34)***. This study used 2D-PAGE and tandem mass spectrometry to perform proteomic analysis on the endometrial carcinoma cell line, Ishikawa, treated with previously reported physiologically attainable genistein concentrations, 0.5, 5, and 50 μ*M* ***(18–22)*** and the supra-physiological concentration, 500 μ*M*. This study enabled the direct measurement of proteins in terms of their presence and relative abundance.

2D-PAGE gels typically contained approx 1800 protein spots, with the majority appearing between pH range 4.0 and 7.0 and M_r of 25 to 70 kDa. A total of 97 proteins displayed significant differential expression following genistein treatment at various concentrations, with 65 protein spots displaying a significant decrease in expression, and 32 protein spots identified as showing a significant increase in

Table 2
Identification of Proteins in Ishikawa Cells That Displayed Regulation in Expression With Genistein Treatment 0.5, 5, 50, or 500 μ*M*[a]

Protein identification	Accession	No. peptides sequences	Sequence coverage	Genistein treatment 0.5 μ*M*	5 μ*M*	5 μ*M*	500 μ*M*	Theoretical $1M_r$/pI	Observed M_r/pI
Calreticulin	gi|4757900	3	16	1.7 ↓*	1.5 ↑*	2.5 ↑*	1.9 ↑*	60/4.3	69/3.2
Heterogeneous nuclear ribonucleoprotein (hnRNP) C	gi|13435678	3	35	1.8 ↓*	2.4 ↓*	1.6 ↓	1.9 ↓	33/5.3	39/4.2
Heat shock 27kD protein 1 (HSP27)	gi|4504517	3	29	2.3 ↓*	2.7 ↓*	3.7 ↓	1.8 ↓	22/6.2	28/5.2
Endoplasmic reticulum protein 29 precursor (ERp29)	gi|5803013	3	29	1.3 ↓	1.4 ↓*	2.0 ↓*	2.6 ↓*	29/7.3	28/6.0
Chaperone containing t-complex polypeptide1 (TCP1) subunit 2	gi|5453603	5	42	1.1 ↓	1.7 ↓*	2.2 ↓*	2.3↓*	57/6.0	55/6.2
Heat shock conjugate 71 kDa (HSC70)	gi|123644	3	11	2.2 ↑	2.9 ↑*	3.1 ↑*	1.6 ↑	33/5.3	39/4.2
Ras-GTPase activating protein	gi|5031703	3	28	2.2 ↑	2.9 ↑*	3.1 ↑*	1.6 ↑	52/5.2	39/4.2
GTP binding protein	gi|4092054	2	39	1.1 ↑	3.9 ↓*	3.3 ↓*	ND[d]	25/9.3	26/7.5
Far upstream element (FUSE) binding protein 1	gi|16878077	1 (58)[b]	15	1.2 ↓	2.7 ↓*	1.6 ↓*	1.0	69/7.4	68/7.4
Rho GDP dissociation inhibitor (GDI) alpha	gi|4757768	1 (56)[b]	35	1.9 ↑	2.3 ↓*	2.1 ↓*	15.2 ↓	23/4.8	29/4.45

(Continued)

Table 2 *(Continued)*

Protein identification	Accession	No. peptides sequences	Sequence coverage	Genistein treatment				Theoretical $1M_r$/pI	Observed M_r/pI
				0.5 μ*M*	5 μ*M*	5 μ*M*	500 μ*M*		
Transcriptional factor 2 (E2F)	gi|15220994	1	17	1.1 ↑	2.1 ↓*	2.1 ↓	6.4 ↓*	45/5.9	40/6.2
hnRNP H2	gi|9624998	2	11	1.0	1.4 ↓	2.2 ↓*	7.7 ↓*	49/5.2	25/6.45
Uracil DNA glycosylase (UDG)	gi|35053	2	36	1.1 ↓	2.3 ↓	3.3 ↓*	ND*[d]	36/8.9	37/9.1
Poly(rC) binding protein 2 (PCBP2)	gi|6997239	2	22	1.3 ↑	1.4 ↓	2.4 ↓*	12.5 ↓*	37/6.3	43/6.5
HnRNP M	gi|14141154	b	24	2.1 ↓	2.2 ↓	1.9 ↓	5.0 ↓*	74/9.0	63/6.6
Enolase 1; phosphopyruvate hydratase	gi|4503571	4	27	1.2 ↓	1.3 ↓	1.6 ↓	1.6 ↓*	47/4.3	48/6.2
Prohibitin	gi|4505773	c	48	1.0	1.2 ↑	1.1 ↑	1.8 ↓*	30/5.4	31/5.2
Heat shock protein 27 (HSP 27)	gi|662841	3	42	1.1 ↑	1.2 ↑	1.3 ↑	2.4 ↓*	22/6.2	28/5.2

[a]Significantly regulated levels ($p < 0.05$) are indicated with asterisk.
[b]Mascot score.
[c]Identified using peptide mass fingerprint.
[d]Spot not present on 500 μ*M* genistein-treated gel.

expression. Of these spots, 29 were randomly selected and excised from the gel to be characterized, from which 18 different proteins were unequivocally identified.

This study demonstrated that treatment with physiologically achievable or supra-physiological concentrations of genistein, regulated five proteins, PCBP2, FUSE, hnRNP C, hnRNP M, and hnRNP H2, involved in modulating the expression of c-myc. Temporal c-Myc protein accumulation is essential for normal cell proliferation. It also plays a key role in cellular differentiation, apoptosis and cell cycle. C-myc is also an oncogene and over expression of Myc protein is known to lead to neoplastic progression, whereas depletion results in inhibition of DNA splicing and subsequently translation of proteins ***(35–38)***. There are several reports showing elevated c-myc gene and protein expression or amplification in premalignant and/or malignant lesions of the endometrium, with increased levels correlating to poor prognosis ***(39–42)***.

The expression of FUSE binding protein was downregulated following treatment with 5 or 50 μ*M* genistein. This protein binds to active c-myc genes and is necessary for c-myc expression. Two members of the hnRNP family, hnRNP C and hnRNP H2 were also downregulated by 0.5 or 5 μ*M* genistein and 5, 50, or 500 μ*M* genistein respectively, whereas another member, hnRNP M was downregulated following treatment at the supra-physiological genistein concentration of 500 μ*M*. These proteins are part of the RNA splicesome and function to modulate translation at the G2/M phase ***(43)***. hnRNP C has been shown to enhance translation of c-myc mRNA ***(43)***. Using 2D-PAGE technology, elevated levels of human hnRNP A2/B1 in endometrial adenocarcinoma cells, as well as a significant increase in hnRNP F in Ishikawa cells treated with estrogen have been demonstrated ***(44,45)***. Using cDNA microarray analysis we previously observed decreased gene expression of six members of the hnRNP family following treatment of Ishikawa cells with 50 μ*M* genistein (unpublished results). Treatment with 50 or 500 μ*M* genistein downregulated the expression of PCBP2, with 500 μ*M* genistein downregulating expression by more than 10-fold. c-Myc contains internal ribosomal entry sites (IRES) and PCBP2 facilitates in translation of reported RNAs containing IRES. Interaction between PCBP2 and c-myc-IRES-RNA has been demonstrated to result in a threefold increases in activation of IRES ***(46)***. Diminishing levels of this protein leads to significantly decreased ability to translate such RNA, thus resulting in decreased levels of protein expression. Previous studies have shown genistein's ability to decrease c-Myc protein in vivo ***(47)***. Furthermore, genistein has also been shown to suppress c-myc mRNA expression in vitro ***(48,49)***.

GTP binding protein was downregulated following treatment with 5 or 50 μ*M* genistein confirming our cDNA microarray analysis, where a decrease in gene expression of GTP-binding protein following 5 μ*M* genistein treatment was observed ***(50)***. Decreased gene expression was also seen with 50 μ*M* genistein treatment (unpublished results). This protein has been implicated in the Ras-related signal transduction pathway. Rho GDP dissociation inhibitor, an inhibitor of the Rho pathway, and a modulator of GTPase was also downregulated following treatment with 5 or 50 μ*M* genistein. Rho GTPases are prominent participants in malignant transformation and are regulated by the *Ras* pathway ***(51,52)***.

c-Myc protein is stabilized by activation of *Ras* through multiple effector pathways ***(53)***. Proteins of the Ras pathway represent a fundamental component in the control of cellular responses, such as proliferation, differentiation, cell cycle control, gene expression, actin cytoskeleton control and intracellular traffic, and nucleocytoplasmic transport, to many incoming signals and in particular mitogenic

stimuli ***(54,55)***. All of these proteins are able to bind to GTP and GDP and exhibit slow GTPase activity ***(56)***. *Ras* is also described as a prominent participant in malignant transformation and is commonly mutated or amplified in endometrial cancer ***(52,57)***. Inhibition of *Ras* activation, or inhibition of proteins facilitating the expression or function of the oncogene c-myc, could inhibit the progression of oncogenesis.

Genistein treatment with physiologically achievable or supra-physiological concentrations, regulated five chaperone proteins. Chaperone and heat shock proteins play important roles in tumor biology ***(58)***.

HSC70, a molecular chaperone involved in protein folding, was upregulated by treatment with 5 or 50 μ*M* genistein. The molecular chaperone activity may contribute to tumorogenesis, by providing the cancer cell with an opportunity to alter protein activities, in particular components of the cell cycle machinery, kinases and other proteins implicated in tumor progression ***(58)***. The expression of this protein is increased in response to heat, oxidative stress, and hypoxia. Cells with low levels usually undergo apoptosis ***(59,60)***. Increased levels of HSC70 have been reported in 31 cancer cell lines, including Ishikawa, compared to noncancerous control cell lines ***(61)***. HSC70 protein was also expressed in high abundance in 10 tumor cell lines ***(58)***. Furthermore, studies raise the possibility that HSC70 may be directly involved in the modulation of oncogene-mediated transformation ***(62)***.

A member of the small heat shock proteins, HSP27 protein 1, was downregulated by physiological concentration of 0.5 or 5 μ*M*, as well HSP27 was downregulated by supra-physiological concentration, 500 μ*M* genistein. This protein regulates epithelial cell growth and differentiation, wound healing, apoptosis, and cell protection against inflammatory cytotoxicity mediators. Elevated levels inhibit necrotic and apoptotic cell death and have been noted in proliferative epithelial breast cancer cells compared to normal ***(63–66)***. Patients with endometrial breast or ovarian cancer, expressed increased levels of antibodies against HSP27 compared to women with benign tumours, strongly suggesting the presence and increased levels of HSP 27 in their circulation ***(67,68)***. Furthermore, HSP27 expression has previously been reported to be elevated in endometrial adenocarcinoma cells ***(44)***.

Chaperone protein ERp29 was downregulated by treatment with 5, 50, or 500 μ*M* genistein. ERp29 shares high sequence similarity to the protein disulfide isomerase family and is described as playing an important role in processing of secretory proteins within the endoplasmic reticulum and a ubiquitously present ***(69,70)***. ERp29 has previously been displayed to be present in high abundance in five tumor cell lines ***(58)***. Calreticulin, a chaperone for glycoproteins in the endoplasmic reticulum, was upregulated following treatment with 5 or 50 μ*M* genistein, however, treatment with 0.5 μ*M* genistein downregulated expression. This protein also belongs to the protein disulfide isomerase family and may inhibit angiogenesis, suppresses tumor growth and inhibit endothelial cell proliferation ***(69,71–75)***.

Incubation of Ishikawa cells with 5, 50, or 500 μ*M* genistein downregulated the expression of TCP1 protein subunits. Using cDNA microarray analysis, we previously observed a decrease in TCP1 gene expression following treatment of Ishikawa cells with 50 μ*M* genistein (unpublished results). TCP1 proteins are heat shock proteins belonging to stress protein family. They are required for the folding of tubulins, actins as well as other cytosolic proteins, to attain microfilament assembly, hence assisting in cell growth ***(76,77)***. The expression levels of TCP1 is closely correlated with growth rates of mammalian cultured cells, with expression strongly upregulated during growth from G(1)/S transition to early S phase ***(78,79)***. Tumor tissues have shown significantly

higher levels of TCP1 expression, as opposed to nontumor tissue and because it is widely distributed in the cytosol, it may represent a useful tumor marker. A high abundance of TCP1 has also been displayed in 10 tumor cell lines ***(58)***.

The regulation of genes involved in cell cycle, DNA replication, DNA repair, and mitosis involves the function of many proteins, including E2F, UDG, and prohibitin. Using cDNA microarray analysis we previously demonstrated decreased gene expression of E2F and UGD following treatment with 50 μ*M* genistein (unpublished results). In this study, E2F protein expression was downregulated following treatment with 5 or 500 μ*M* genistein. This protein displays RNA polymerase II transcription factor activity with positive regulation of cell cycle activity and has been shown to be associated with several Ras proteins ***(80)***. UDG protein was also downregulated following treatment with 5 or 500 μ*M* genistein. UDG is activated by E2F-1 and removes the uracil moieties from DNA and functions to repair U:G mispairs ***(81)***. Prohibitin, was downregulated following treatment with 500 μ*M* genistein. Prohibitin is a proto-oncogene involved in cell-cycle control and senescence, is known to suppress growth and repress E2F mediated transcription, inhibiting apoptosis and has been shown to be elevated in endometrial adenocarcinoma cells ***(44,82,83)***.

Enolase 1 protein was downregulated following treatment with 500 μ*M* genistein. Using cDNA microarray analysis, we previously observed a decrease in gene expression of enolase 1 following treatment of Ishikawa cells with 50 μ*M* genistein (unpublished results). This protein is involved in glycolysis pathway (ADP to ATP) and has been reported to be over expressed in 20 cancer classes ***(84)***. Furthermore, glycolytic enzymes and alpha enolase were also reported to be elevated in hyperplastic and neoplastic endometrial cells, this finding is believed to be owing to the increase rate of glycolysis ***(44)***. Enolase 1 has previously been described as negative regulator of NF-κB ***(85)***. NF-κB is a ubiquitously expressed, pleiotropic transcription factor, with an important role in the control of cell proliferation, apoptosis and oncogenesis ***(86,87)***. Constitutively activated NF-κB has been postulated as promoting oncogenesis in certain cancers ***(87,88)***. Choi et al. showed that the DNA-binding activity of NF-κB in nuclear extracts treated with 50 and 100 μ*M* genistein was significantly suppressed ***(89)***.

In summary, this study used 2D-PAGE technology to display variations in protein expression of Ishikawa cells following treatment with genistein at varying concentrations. The findings indicate that genistein can regulate the cellular activity of Ishikawa cells, and hence may affect the state of the living cell. Genistein's involvement in either up or downregulating the proteins identified indicates that it may exhibit a global effect on the cell resulting in reduced transcription/activation of known oncogenes or cellular proliferation, and subsequently reduce oncogenesis. The results obtained from this study suggest that genistein regulates the expression of proteins involved in endometrial oncogenesis.

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