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Expression profiling of the estrogen responsive genes in response to phytoestrogens using a customized DNA microarray

Ryota Ise[a], Dalho Han[a], Yusuke Takahashi[b], Shunichi Terasaka[c], Akio Inoue[a], Masao Tanji[a], Ryoiti Kiyama[a,d,*]

[a] *InfoGenes Co., Ltd., Tsukuba, Ibaraki 305-0047, Japan*
[b] *Industrial Research Institute of Tottori Prefecture, Tottori, Tottori 689-1112, Japan*
[c] *SciMedia Ltd., Taito-ku, Tokyo 110-0008, Japan*
[d] *National Institute of Advanced Industrial Science and Technology, Tsukuba, Ibaraki 305-8566, Japan*

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Abstract Here, we examined phytoestrogens, isoflavones (genistein, daidzein, glycitein, biochanin A and ipriflavone), flavones (chrysin, luteolin and apigenin), flavonols (kaempferol and quercetin), and a coumestan, a flavanone and a chalcone (coumestrol, naringenin and phloretin, respectively) by means of a DNA microarray assay. A total of 172 estrogen responsive genes were monitored with a customized DNA microarray and their expression profiles for the above phytoestrogens were compared with that for 17β-estradiol (E_2) using correlation coefficients, or *R* values, after a correlation analysis by linear regression. While *R* values indicate the similarity of the response by the genes, we also examined the genes by cluster analysis and by their specificity to phytoestrogens (specific to genistein, daidzein or glycitein) or gene functions. Several genes were selected from *p53*-related genes (*CDKN1A*, *TP53I11* and *CDC14*), *Akt*2-related genes (*PRKCD*, *BRCA1*, *TRIB3* and *APPL*), mitogen-activated protein kinase-related genes (*RSK* and *SH3BP5*), Ras superfamily genes (*RAP1GA1*, *RHOC* and *ARHGDIA*) and AP-1 family and related genes (*RIP140*, *FOS*, *ATF3*, *JUN* and *FRA2*). We further examined the extracts from two local crops of soy beans (Kuro-daizu or Mochi-daizu) by comparing the gene expression profiles with those of E_2 or phytoestrogens as a first step in utilizing the expression profiles for various applications.

Keywords: DNA microarray; Expression profile; Phytoestrogen; Signal transduction

1. Introduction

Phytoestrogens are chemicals that originate from plants with estrogenic activity. They have attracted significant attention because of their ability to modulate the action of estrogen, which may be used for anti-cancer agents, agents for hormone therapy, dietary additives and supplements, and antimicrobial agents [1–4]. The growing importance of phytoestrogens is partly supported by genetic and genomic evidence of their effects. Understanding more about the gene function networks and signal transduction pathways initiated or mediated by these chemicals, therefore, will be of great benefit to further applications of phytoestrogens.

DNA mircroarrays have been used for the characterization of phytoestrogens to examine genetic responses, to predict effects and to discover new materials and sources [5–14]. One of the advantages of using DNA microarrays is that it allows a comprehensive analysis of gene responses under specific conditions, revealing gene networks and providing significant clues as to their mechanisms of function. Despite concerns raised in some reports about the reliability of data [15–17], increasing the statistical power through repetition of the assay, selecting reliable genes, and refining the statistical approach by adopting a correlation analysis will all improve the results. In this context, gene expression analysis using customized DNA microarrays or focused microarrays is attracting more attention because of these advantages along with a reduction in the cost and time needed for the analysis (reviewed in [18]). Statistical evaluation partly but significantly contributes to the use of focused microarrays. The data obtained are incorporated into databases and will be used for the classification and screening of genes and for giving annotations to genes for further application of the data for mining useful information about target genes and products, signaling pathways and their outcomes (reviewed in [19–21]).

We previously developed a customized DNA microarray containing estrogen responsive genes from human cells and used it to analyze the effects of natural estrogens, antagonists of estrogens and industrial estrogenic chemicals [14,22]. Using the DNA microarray, we characterized phytoestrogens to better understand the effects, mechanisms and outcomes of their actions in human cells.

*Corresponding author. Fax: +81 029 859 5135.
E-mail address: kiyama.r@aist.go.jp (R. Kiyama).

Abbreviations: E_2, 17β-estradiol; GEN, genistein; DAI, daidzein; GLY, glycitein; BIO, biochanin A; CHR, chrysin; LUT, luteolin; API, apigenin; IPR, ipriflavone; KAE, kaempferol; QUE, quercetin; COU, coumestrol; NAR, naringenin; PHL, phloretin; MAPK, mitogen-activated protein kinase; SSC, 0.15 M NaCl and 0.015 M sodium citrate

2. Materials and methods

2.1. Chemicals

Natural estrogen, 17β-estradiol (E_2), genistein (GEN) and chrysin (CHR) were purchased from Sigma–Aldrich (St. Louis, MO, USA) and daidzein (DAI) was obtained from Nacalai Tesque (Kyoto, Japan). Glycitein, biochanin A (BIO), luteolin (LUT), apigenin

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(API), ipriflavone (IPR), coumestrol (COU), kaempferol (KAE), quercetin (QUE), naringenin (NAR) and phloretin (PHL) were obtained from Wako Pure Chemical Industries (Osaka, Japan). A 10 mM stock of all compounds was dissolved in ethanol and the final concentration of solvent in the culture medium did not exceed 0.1%; this concentration did not affect cell yields. Fetal bovine serum (FBS) was purchased from HyClone (Logan, UT, USA). Penicillin–streptomycin solution and trypsin/EDTA solution were all purchased from Life Technologies (Grand Island, NY, USA). Soy beans (Kuro-daizu and Mochi-daizu; gifts from Fujicco Co., Ltd. and Tottori Agricultural Experiment Station, respectively) were homogenized in 15 mM Tris buffer (pH 8) using a rolling mill at a ratio of 1 g of soy bean per 10 ml of buffer. The resulting Tris homogenate was centrifuged twice at 15000 rpm for 10 min, and the supernatant was collected and extracted with an equal volume of acetone. The acetone extracts were dried under a stream of nitrogen and the residue was dissolved in 1 ml of acetone, and stored at −20 °C prior to use. These soy bean extracts were used at a concentration of 1 mg/ml in ethanol. All other chemicals and biochemicals were of the highest quality available from commercial sources.

2.2. Cell culture and RNA extraction

Human breast cancer MCF-7 cells were obtained from JCRB Cell Bank (National Institute of Health Sciences, Tokyo, Japan) and routinely cultured in RPMI 1640 medium (Invitrogen, Carsbad, CA, USA) supplemented with 10% FBS at 37 °C in a humidified atmosphere of 95% air and 5% CO_2. Cells were cultured in phenol red-free RPMI 1640 medium containing 10% dextran-coated charcoal-treated FBS (DCC-FBS) for 6 days and treated with 10 nM of E_2 or control (0.1% ethanol) vehicle and 10 μM of phytoestrogens for 3 days. After the cells were cultured for 3 days in the presence of chemicals at specific concentrations, mRNA was purified using the PolyATract System 1000 (Promega, Madison, WI, USA) according to the manufacturer's instructions. The quality of mRNA was confirmed by spectrophotometry.

2.3. cDNA microarray analysis

A custom cDNA microarray (EstrArray) was manufactured by InfoGenes (Tsukuba, Japan) by mechanical spotting of the cDNA (~500 to ~1.5 kb) of selected estrogen responsive genes as described previously [14]. For the EstrArray assay, biologically independent DNA microarray assays were repeated five times for E_2, genistein, daidzein and glycitein, and twice for the other phytoestrogens. The analysis using EstrArrays was performed as follows: each mRNA was labeled with fluorescent Cyanine 3 (Cy3)-dUTP for the treatment of chemicals or Cyanine 5 (Cy5)-dUTP for the control vehicle at 42 °C for 80 min using Superscript II (Invitrogen) and random primers (a mixture of 6 mers and 9 mers). Both Cy3- and Cy5-labeled probes were mixed and denatured under alkaline conditions for 1 h. After free fluorescent nucleotides were removed using CyScribe GFX Purification kit (Amersham Bioscience, Buckinghamshire, UK), probes were hybridized to EstrArrays for 16 h in Hybridization buffer [5× 0.15 M NaCl and 0.015 M sodium citrate (SSC) containing 0.5% SDS] at 65 °C. After hybridization, slides were washed in 2× SSC/0.2% SDS for 5 min at

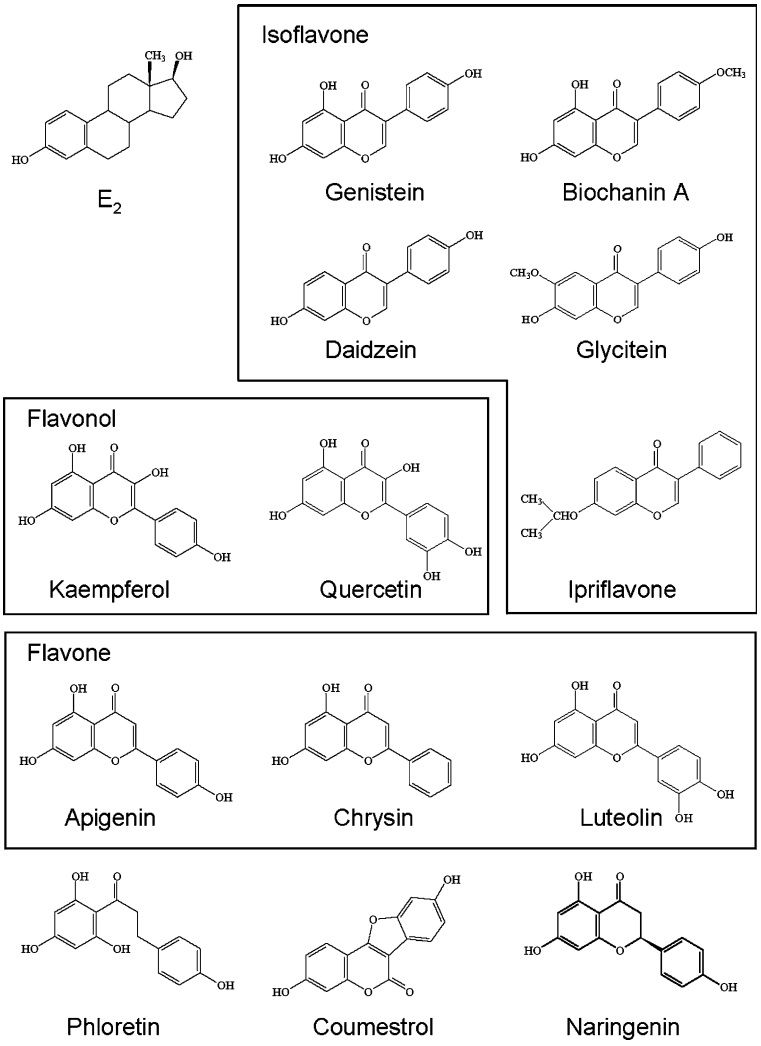

Fig. 1. Chemical structures of phytoestrogens analyzed here. Chemical formulas were modified to show the similarity to E_2.