

Comparison of the Effects of Phenethyl Isothiocyanate and Sulforaphane on Gene Expression in Breast Cancer and Normal Mammary Epithelial Cells

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Phenethyl isothiocyanate (PEITC) and sulforaphane (SF) exhibit tumor preventive activity in lung, prostate, breast and colon cancers. Our objective was to examine the effect of these two isothiocyanates on estrogen receptor-related genes, and genes related to apoptosis and cell cycle in the estrogen-dependent breast cancer cell line MCF7 and in normal human epithelial breast (HME) cells. We treated cells with 0.3 μ M or 3.0 μ M concentrations of PEITC or SF. In HME cells, gene expression was significantly altered for 23 genes by PEITC at a concentration of 0.3 μ M and 4 genes at 3.0 μ M. SF altered the expression of 16 genes at a concentration of 0.3 μ M and 2 genes at 3.0 μ M. In HME cells, genes altered by both PEITC and SF exhibited changes in gene expression that were similar in extent as well as direction of change. In MCF-7 cells, PEITC did not produce any significant changes in the gene expression at both treatment levels. SF produced significant changes in 7 genes, but only at the higher treatment level of 3.0 μ M. Normal mammary cells exhibited more changes in the expression of estrogen receptor related genes than did breast cancer cells, and significantly these changes occurred predominantly at the low concentration of 0.3 μ M, a concentration achievable by dietary input of isothiocyanates. Novel findings were the upregulation of the pro-apoptotic gene BAD and estrogen receptor beta gene in normal human mammary cells. These gene alterations observed, along with upregulation of tumor suppressors p21 and p27, may provide a protective effect to mammary cells against breast cancer. *Exp Biol Med* 234:287–295, 2009

Key words: phenethyl isothiocyanate; sulforaphane; human

mammary epithelial cells; breast cancer MCF-7 cells; breast cancer prevention; gene expression

Introduction

The inverse correlation between consumption of cruciferous vegetables and cancer risk has been demonstrated in lung, colon, breast, stomach and prostate cancers (1–3). Organic isothiocyanates, one of the components of cruciferous vegetables, have been identified as a class of compounds that may cause this effect. Isothiocyanates (general chemical structure $R-N=C=S$) occur in crucifers as glucosinolate precursors. Isothiocyanates inhibit the metabolic activation of a variety of carcinogens that occur in tobacco products and the diet (4). More recent studies have uncovered additional pathways such as induction of apoptosis which can explain the anti-carcinogenic actions of isothiocyanates (5). Sulforaphane (SF) and phenethyl isothiocyanate (PEITC) are two organ isothiocyanates obtained mainly from broccoli and watercress, respectively (6). An epidemiologic study with 720 breast cancer cases and 810 controls reported that the consumption of broccoli is inversely associated with breast cancer risk in premenopausal women (1). In vitro, PEITC is cytotoxic in human breast cancer MCF-7 and human mammary epithelial MCF-12A cells, with IC_{50} values of $7.32 \pm 0.25 \mu$ M and $7.71 \pm 0.07 \mu$ M, respectively (7). A maximal plasma concentration of approximately 1.0 μ M is achieved by consumption of 100 grams of watercress (8). In order to investigate the mechanisms by which these ITCs can exert preventive and cytotoxic effects in breast cancer, we used a human breast cancer gene array with 96 genes (SuperArray Inc.) to assess changes in gene expression.

Most in vitro experiments have so far focused on the effect of high treatment levels of ITCs on cancer cells. However, the most likely exposure of isothiocyanates is exposure to low levels in human beings who do not have

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Table 1. Genes Altered by Isothiocyanate Treatment in Mammary Cells

Treatment level	HME cells	MCF7 cells
0.3 μ M PEITC	23	-
3.0 μ M PEITC	4	-
0.3 μ M sulforaphane	16	-
3.0 μ M sulforaphane	2	7

breast cancer. The effect of isothiocyanates on mammary cells in such an environment, to our knowledge, has not been studied. Our objective was to study the effect of two common dietary ITCs, PEITC and SF, on gene expression changes in normal and cancerous human mammary epithelial cells, and to determine chemopreventive potential of isothiocyanates in breast cancer. Our hypothesis is that the changes in gene expression between normal and breast cancer cell lines are distinct and these changes may be important when evaluating the “protective” effects of isothiocyanates. Changes in gene expression may be able to explain the apparent protective effects of isothiocyanates against breast cancer.

Materials and Methods

Materials. Mammary Epithelial Basal Medium, epidermal growth factor, hydrocortisone, insulin, and bovine pituitary extract were purchased from Cambrex Corp. (these items are now available from Lonza Inc., Walkersville, MD). Transferrin, isoproterenol and dimethyl sulfoxide were obtained from Sigma Aldrich. PEITC and SF were obtained from LKT Laboratories (St. Paul, MN). RPMI1640, penicillin, streptomycin, fetal bovine serum and MMLV reverse transcriptase were obtained from Invitrogen (Grand Island, NY). GEArray Q series Estrogen Receptor Signaling Gene Arrays and Ampolabeling LPR (linear polymerase reaction) kit were obtained from SABiosciences Inc. (Frederick, MD). SV RNA isolation kit was obtained from Promega Corporation (Madison, WI). MCF7 cells were provided by Dr. Susan E. Bates (National Cancer Institute, Bethesda, MD) and HME cells were provided by Dr. Martha Stampfer (Lawrence Berkeley National Laboratory, Berkeley, CA). The HME cells represent normal finite lifespan mammary cells which were obtained from reduction mammoplasty tissue of a 21-year-old woman.

Methods. Cell Culture. HME cells were incubated in 75 ml flasks until 60–80% confluence in Mammary Epithelial Basal Medium supplemented with 5 ng/ml epidermal growth factor, 500 ng/ml hydrocortisone, 5 μ g/ml insulin, 70 μ g/ml bovine pituitary extract, 5 μ g/ml transferrin, and 10^{-5} M isoproterenol at 1% CO_2 in a 37°C incubator. The cells were treated with dimethyl sulfoxide 0.015% v/v (control), SF or PEITC at one of two concentrations (0.3 μ M or 3.0 μ M) for 48 hours ($n = 3$). These concentrations are similar to the plasma levels of

PEITC that can be achieved after ingestion of 100 gm of watercress (8). MCF-7 cells in 75 ml flasks were incubated in RPMI1640 supplemented with 100 units/ml penicillin, and 100 μ g/ml of streptomycin and 10% fetal bovine serum until 60–80% confluence at 5% carbon dioxide in a 37°C incubator. Cells were treated with dimethyl sulfoxide 0.015% v/v (control), SF or PEITC at one of two concentrations (0.3 or 3.0 μ M) for 48 hours. At the end of the incubation period, all cells except HME cells at 3.0 μ M were harvested using ice-cold phosphate buffer saline and cell scraping. HME cells at 3.0 μ M were harvested via direct application of lysis buffer from the RNA isolation kit.

RNA Isolation and cDNA Formation. Total RNA was isolated from both cell lines by using the SV RNA Isolation System and quantified spectrophotometrically at 260 nm. cDNA was prepared from total RNA by reverse transcription with MMLV reverse transcriptase or using ampolabeling LPR kit.

Hybridization and Imaging. GEArray Q series Estrogen Receptor Signaling Gene Arrays were employed according to the manufacturer's instructions. Ninety-six genes were used to study the expression profile of the genes involved in estrogen receptor signaling pathways. cDNA was chemiluminescence-labeled using biotin, hybridized under precisely specified conditions to a positively charged nylon membrane containing the arrayed DNA. After washing, the relative expression level of each gene was analyzed using a Kodak Image Station 440CF.

Normalization and Statistical Analysis. The mean intensity for each gene spot was measured. The mean intensity of the background PUC18/Blank genes was subtracted from mean intensity to give net mean intensity. The average of total intensity on the array was used to normalize the intensity of the gene spots. The average intensity for a gene for each treatment was compared with the control group, with the Student's *t* test, with the level of significance at 0.05. Significance Analysis of Microarrays (SAM) was also used to analyze the data, which accounts for errors arising from repeated measurements (9). While using SAM, delta was set such that the false discovery rate for each array was minimized. The false detection rate for comparisons ranged from 0–1%. Results from both tests were compared, and genes that were significant by both tests are reported.

Results

As shown in Table 1, in normal as well as cancer cells, isothiocyanates produced significant gene expression changes in a number of genes. In HME cells, gene expressions were significantly altered for 23 genes by PEITC at a concentration of 0.3 μ M and 4 genes at a concentration of 3.0 μ M. SF altered the expression of 16 genes at 0.3 μ M and 2 genes at 3.0 μ M. In HME cells, genes altered by both PEITC and SF exhibited changes in gene expression that were similar in extent (fold change) as well

as direction of change (up- or downregulation). In MCF-7 cells, PEITC did not produce any significant changes in the gene expression at both treatment levels. SF produced a significant change in 7 genes, only at the higher treatment level of 3.0 μM in MCF-7 cells. Isothiocyanates altered the expression of more genes in human mammary epithelial cells than breast cancer cells. Genes altered were related to (i) apoptosis and cell cycle regulation (example: BAD, p21, p27), (ii) cell adhesion (example: claudin-7, fibronectin), (iii) estrogen receptor signaling (example: estrogen receptor beta) and (iv) prognostic cancer markers (example: her2, EGFR). Genes significantly affected by treatment are listed in Table 2. A listing of the genes present in the GEArray Q Series Estrogen Receptor Signaling Gene Array can be found in the Appendix.

Discussion

Isothiocyanates are compounds derived from cruciferous vegetables such as broccoli, cabbage and watercress. Based on epidemiological studies, isothiocyanates are widely recommended as cancer preventive agents and commercially available in herbal supplements (1, 10, 11). The purpose of this study was to evaluate these isothiocyanates as breast cancer preventive agents. Breast cancer progression is determined by changes in cellular proliferation, apoptosis and metastasis. The estrogen receptor signaling pathway in estrogen receptor positive breast epithelial cells provides an additional mechanism for cellular proliferation (12). We examined the effects of PEITC and SF on expression of genes related to estrogen receptor signaling and other pathways important in the development of breast cancer using primary cultures of normal human mammary epithelial cells and the cancerous estrogen receptor-positive breast cancer cell line MCF7. Comparing the effects of ITCs on normal versus cancer cell lines will provide insight in their role in the prevention of breast cancer.

Genes Related to Apoptosis and Cellular Proliferation. Mechanisms of cancer prevention include the induction of apoptosis and reduction of cellular proliferation. Isothiocyanates have been reported to induce apoptosis and reduce cellular proliferation in a number of cancer cell lines in vitro (13, 14). We report for the first time induction of BAD, a pro-apoptotic gene by 0.3 μM PEITC in mammary cells. A time-dependent upregulation of BAD, along with increase in apoptosis has been observed in pre-adipocyte cell line AML-I treated with 100 $\mu\text{mol/L}$ quercetin, a dietary flavonoid (15). BAD belongs to the Bcl-2 family of proteins and regulates apoptosis (16, 17). BAD is downregulated completely by estradiol in MCF7 cells (18). Isothiocyanates have been shown to upregulate the related protein BAX in vitro as well as in vivo (19, 20). Other genes reported to have pro-apoptotic effects including THSB4 and GADD34 were also upregulated by PEITC. GADD34 is reported as a pro-apoptotic growth suppressor.

Other GADD family members have been reported to be induced by 25 μM PEITC treatment in human adenocarcinoma HCT-116 cells (21).

Anti-apoptotic and proliferative effects observed include upregulation of v-jun and Ki67. V-jun, a known oncogene, was upregulated by both PEITC and SF. ITC-induced activation of AP-1 pathway (of which v-jun is a member) has previously been reported. Concentrations of 5–10 μM PEITC and SF produce significant activation of AP-1 activity as measured by luciferase activity assay when incubated with the prostate cancer cell line PC3-C9 and the bladder cancer cell line UM-UC-3 (22, 23). Ki67, a cell proliferation marker associated with apoptosis, was upregulated by both PEITC and SF at 0.3 μM concentrations.

Metallothionein 3 (MT3) was significantly upregulated by PEITC. While overexpression of this gene has been shown to inhibit growth of certain breast cancer cell lines including MCF7, expression of MT3 has also been correlated to higher grade tumors and poor cancer prognosis (24, 25).

To summarize, isothiocyanates induced cyclin-dependent kinase inhibitors, but other effects, including the induction of Ki67 and v-Jun, make it difficult to determine if induction of cyclin-dependent kinase inhibitors will result in apoptosis. In HME cells, SF induces anti-apoptosis, but no pro-apoptosis or proliferation genes; therefore, PEITC may have greater effects than SF on the induction of apoptosis in mammary cells.

Genes Related to Cell Adhesion. Cell adhesion is an important feature of cellular scaffolding, which prevents cells from breaking away from the existing tissue and migrating to another location. A decrease in the adhesion molecules such as E-cadherin has been shown to be correlated with increased metastasis, while fibronectin can increase cellular motility of breast cancer cells (26, 27). In the present study, we found that a number of cell adhesion molecules were altered by isothiocyanate treatment. Following treatment with 0.3 μM PEITC or SF, both altered motility facilitating cell adhesion molecules (fibronectin (FLRT1), claudin-7, integrin-b4 and episialin by PEITC and CD44 by SF). Claudin-7 expression is reported to be lower in invasive carcinomas than in normal breast tissue, and knockdown of claudin-7 expression leads to loss of E-cadherin and increased invasiveness in squamous cell carcinoma cells. Promotion of cell motility and enhancement of metastatic features may signal a harmful effect of isothiocyanates. The role of isothiocyanates in the loss of cell adhesion has previously been reported; 12 μM allyl isothiocyanate has been shown to increase cell detachment in HT-29 colorectal cells (28, 29).

Estrogen Receptor and Gene Interactions. Estrogen receptors alpha and beta play an important role in the regulation of cell growth and proliferation in mammary cells. Several studies have shown that the actions of these two receptors are often opposing. ERalpha may enhance cellular proliferation of MCF7 cells while the addition or

Table 2. Effect of Isothiocyanates on Gene Expression in Human Mammary Epithelial (HME) and Human Breast Cancer MCF-7 Cells

GeneBank number	Gene description	Fold change
0.3 μM PEITC on HME cells		
NM_003248	Thrombospondin 4 ^a	Inf
NM_013280	Fibronectin leucine rich transmembrane protein 1	147.3
NM_002581	Pregnancy-associated plasma protein A	38.42
NM_005954	Metallothionein 3 (growth inhibitory factor (neurotrophic))	30.52
NM_004322	BCL2-antagonist of cell death	24.08
NM_005438	FOS-like antigen 1	22.95
NM_001237	Cyclin A2	22.36
NM_182741	Mucin 1, transmembrane	21.88
NM_002228	V-jun sarcoma virus 17 oncogene homolog (avian)	21.20
NM_000044	Androgen receptor (dihydrotestosterone receptor; testicular feminization; spinal and bulbar muscular atrophy; Kennedy disease)	19.86
NM_004448	V-erb-b2 erythroblastic leukemia viral oncogene homolog 2, neuro/glioblastoma derived oncogene homolog (avian)	17.94
NM_004064	Cyclin-dependent kinase inhibitor 1B (p27, Kip1)	14.79
NM_002417	Antigen identified by monoclonal antibody Ki-67	13.01
NM_002407	Secretoglobulin, family 2A, member 1	11.94
NM_014330	Protein phosphatase 1, regulatory (inhibitor) subunit 15A (GADD34)	7.923
NM_005694	COX17 homolog, cytochrome c oxidase assembly protein (yeast)	7.829
NM_001437	Estrogen receptor 2 (ERbeta)	7.555
NM_003486	Solute carrier family 7 (cationic amino acid transporter, y+ system), member 5	5.594
	CD98	
NM_000389	Cyclin-dependent kinase inhibitor 1A	5.281
	P21/Waf1/CIP1	
NM_002284	Keratin, hair, basic, 6 (monilethrix)	4.344
NM_000213	Integrin, beta 4	3.981
NM_001993	Coagulation factor III (thromboplastin, tissue factor)	-2.355
NM_001909	Cathepsin D (lysosomal aspartyl protease)	-8.045
NM_001307	Claudin 7	-26.04
3.0 μM PEITC on HME cells		
NM_001993	Coagulation factor III (thromboplastin, tissue factor)	-3.937
NM_003236	Transforming growth factor, alpha	-3.279
NM_021130	Homo sapiens peptidylprolyl isomerase A (cyclophilin A) (PPIA)	-5.102
NM_005978	S100 calcium binding protein A2 (CaN19)	-2.096
0.3 μM SF on HME cells		
NM_000600	Interleukin 6 (interferon, beta 2)	61.51
NM_005954	Metallothionein 3 (growth inhibitory factor (neurotrophic))	37.98
NM_002581	Pregnancy-associated plasma protein A	34.34
NM_182741	Mucin 1, transmembrane	22.96
NM_002228	V-jun sarcoma virus 17 oncogene homolog (avian)	22.06
NM_000044	Androgen receptor (dihydrotestosterone receptor; testicular feminization; spinal and bulbar muscular atrophy; Kennedy disease)	19.88
NM_004448	V-erb-b2 erythroblastic leukemia viral oncogene homolog 2, neuro/glioblastoma derived oncogene homolog (avian)	16.76
NM_003247	Thrombospondin 2	15.20
NM_002407	Secretoglobulin, family 2A, member 1	9.470
NM_003226	Trefoil factor 3 (intestinal)	9.378
NM_001437	Estrogen receptor 2 (ERbeta)	7.882
NM_005694	COX17 homolog, cytochrome c oxidase assembly protein (yeast)	7.078
NM_000389	Cyclin-Dependent Kinase Inhibitor 1A	6.606
NM_002284	Keratin, hair, basic, 6 (monilethrix)	5.224
NM_000610	CD44 antigen (homing function and Indian blood group system)	2.391
NM_005228	Epidermal growth factor receptor (erythroblastic leukemia viral (v-erb-b) oncogene homolog, avian)	2.380
3.0 μM SF on HME cells		
NM_001993	Coagulation factor III (thromboplastin, tissue factor)	-4.950
NM_001909	Cathepsin D (lysosomal aspartyl protease)	-1.923
3.0 μM SF on MCF7 cells		
NM_000077	Cyclin-dependent kinase inhibitor 2A (melanoma, p16, inhibits CDK4)	19.13

Table 2. Continued.

GeneBank number	Gene description	Fold change
NM_003246	Thrombospondin 1	10.11
NM_000610	CD44 antigen (homing function and Indian blood group system)	9.601
NM_000988	Ribosomal protein L27	2.870
NM_021130	Homo sapiens peptidylprolyl isomerase A (cyclophilin A) (PPIA)	1.642
NM_003486	Solute carrier family 7 (cationic amino acid transporter, y+ system), member 5	-6.11
NM_002051	GATA binding protein 3	-13.70

^a Thrombospondin 4 did not have any signal in the controls, while it showed signal in the treated group, leading to values of “infinity” in fold

endogenous expression of ERbeta by these cells reduces their proliferation by causing cell cycle arrest in G2 phase (30). We saw an increase in ERbeta gene expression in HME cells following treatment with 0.3 μ M of PEITC and SF. This was accompanied by increased expression of p21 and p27 mRNA. A less than 5-fold increase on p21 and p27 protein was observed on treatment of PC-3 cells with indole-3-carbinol for 48 hours (31). Comparable treatments of 30 μ M showed significant reductions in cell proliferation under the same conditions. The effect of ERbeta on gene expression of these two tumor suppressor genes has been shown previously in MCF7 cells (30). Cyclin A and Ki67, which were upregulated by 0.3 μ M PEITC in our study, have been shown to be elevated by ERbeta expression (32). Cyclin A peaks in the G2 phase, further indicating that ITCs, especially PEITC, may be causing G2/M phase arrest in HME cells at low treatment levels. G2 arrest through the upregulation of genes such as ERbeta, p21 and p27 may represent a mechanism of cancer prevention of PEITC.

Prognostic Cancer Markers. The upregulation of Her2 by PEITC and co-upregulation of her2 and EGFR by SF in normal breast cells may be a matter of concern when evaluating ITC effects. These effects may represent a “pro-cancerous” effect of ITCs if not overshadowed by their other beneficial effects, such as p21 upregulation. More studies of effects of ITCs on cell cycle and her2 in breast cells are necessary to determine their effects in breast cancer prevention.

Gene Expression Changes Dependent on Concentration and Cell Type. MCF7 cells appear to be more resistant to gene expression changes than HME cells, which may mean that some of the regulatory mechanisms responsible for these changes may be transformed in the conversion of normal to mammary cancer cells.

IC50s of SF and PEITC in MCF7 cells are known and the concentrations of treatment are much lower than IC50s of these compounds. (7) However, IC50s in HME cells have not been investigated. It is possible that the ITCs are more cytotoxic to HME cells and therefore produce more changes in apoptosis and cell proliferation-related cells. However, this needs to be investigated.

Additionally, it is observed that lower concentrations of isothiocyanates exhibit a different gene expression pattern than the higher concentrations; metabolism may contribute

to the changes observed. Part of the changes that we see may be caused by active metabolites of isothiocyanates. Induction of apoptosis in human alveolar basal epithelial cells A549 by phenethyl isothiocyanate-N acetylcysteine has been demonstrated (33). The ratios of metabolites to parent may change depending on starting concentrations of the isothiocyanates, and this may lead to different genes being affected. Assessment of metabolism of ITCs in HME cells may provide explanations for the apparent differences in expression.

Conclusion. Overall, isothiocyanates have numerous effects on gene expression in human mammary cells. A very significant finding was the greater number of changes in gene expression observed at dietary concentrations of isothiocyanates (0.3 μ M) compared with that observed following a 10-fold higher concentration. Several effects observed in HME cells are consistent with reports of ITCs effects in other cancer cell lines. Novel findings were the upregulation of the pro-apoptotic gene BAD and estrogen receptor beta gene in normal human mammary epithelial cells. These gene alterations observed, along with upregulation of tumor suppressors p21 and p27, may provide a protective effect to mammary cells against breast cancer. However, we need to be cautious about the net effects of isothiocyanates as several other alterations, such as upregulation of her2 and EGFR, may present unfavorable effects of isothiocyanates. These need to be investigated in order to further understand the effects of isothiocyanates on breast cancer. Additional studies such as determining contribution of metabolites and parent compounds to apoptosis and cellular adhesion in normal versus cancer cell lines up on treatment of cells at these concentrations will be helpful in determining the final effects of these treatments on the cells.

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Appendix. Layout of GEArray Q Series Human Breast Cancer and Estrogen Receptor Signaling Gene Array
(from SuperArray, Inc.)

Position	GeneBank	Symbol	Description	Gene name
1	NM_000044	AR	Androgen receptor (dihydrotestosterone receptor; testicular feminization; spinal and bulbar muscular atrophy; Kennedy disease)	AR
2	NM_001185	AZGP1	Alpha-2-glycoprotein 1, zinc	AZGP1
3	NM_004322	BAD	BCL2-antagonist of cell death	Bad
4	NM_004323	BAG1	BCL2-associated athanogene	BAG-1
5	NM_000633	BCL2	B-cell CLL/lymphoma 2	Bcl-2
6	NM_004050	BCL2L2	BCL2-like 2	Bcl-w
7	NM_015548	BPAG1	Bullous pemphigoid antigen 1, 230/240kDa	BPAG1
8	NM_000064	C3	Complement component 3	C3
9	NM_003914	CCNA1	Cyclin A1	Cyclin A1
10	NM_001237	CCNA2	Cyclin A2	Cyclin A
11	NM_053056	CCND1	Cyclin D1 (PRAD1: parathyroid adenomatosis 1)	Cyclin D1
12	NM_001238	CCNE1	Cyclin E1	Cyclin E1
13	NM_004702	CCNE2	Cyclin E2	Cyclin E2
14	NM_000610	CD44	CD44 antigen (homing function and Indian blood group system)	CD44
15	NM_004360	CDH1	Cadherin 1, type 1, E-cadherin (epithelial)	E-cadherin
16	NM_000389	CDKN1A	Cyclin-dependent kinase inhibitor 1A	P21/Waf1/CIP1
17	NM_004064	CDKN1B	Cyclin-dependent kinase inhibitor 1B (p27, Kip1)	p27Kip1
18	NM_000077	CDKN2A	Cyclin-dependent kinase inhibitor 2A (melanoma, p16, inhibits CDK4)	p16INK4
19	NM_001307	CLDN7	Claudin 7	CLDN7
20	NM_001831	CLU	Clusterin (complement lysis inhibitor, SP-40,40, sulfated glycoprotein 2, testosterone-repressed prostate message 2, apolipoprotein J)	TRPM2/SP-40/APOJ
21	NM_001848	COL6A1	Collagen, type VI, alpha 1	COL6A1
22	NM_005694	COX17	COX17 homolog, cytochrome c oxidase assembly protein (yeast)	COX17
23	NM_001904	CTNNB1	Catenin beta 1	b Catenin
24	NM_001908	CTSB	Cathepsin B	Cathepsin B
25	NM_001909	CTSD	Cathepsin D (lysosomal aspartyl protease)	Cathepsin D
26	NM_000103	CYP19A1	Cytochrome P450, family 19, subfamily A, polypeptide 1	ARO1
27	NM_006094	DLC1	Deleted in liver cancer 1	DLC1
28	NM_005228	EGFR	Epidermal growth factor receptor (erythroblastic leukemia viral (v-erb-b) oncogene homolog, avian)	EGFR
29	NM_004448	ERBB2	V-erb-b2 erythroblastic leukemia viral oncogene homolog 2, neuro/glioblastoma derived oncogene homolog (avian)	TKR1/Her-2
30	NM_000125	ESR1	Estrogen receptor 1	ER alpha
31	NM_001437	ESR2	Estrogen receptor 2 (ERbeta)	ER-beta-cx
32	NM_001993	F3	Coagulation factor III (thromboplastin, tissue factor)	TF
33	NM_000800	FGF1	Fibroblast growth factor 1 (acidic)	FGF1
34	NM_013280	FLRT1	Fibronectin leucine rich transmembrane protein 1	FLRT1
35	NM_005438	FOSL1	FOS-like antigen 1	Fra-1
36	NM_014211	GABRP	Gamma-aminobutyric acid (GABA) A receptor, pi	GABRP
37	NM_002051	GATA3	GATA binding protein 3	GATA3
38	NM_080425	GNAS	GNAS complex locus	GNAS1
39	NM_000177	GSN	Gelsolin (amyloidosis, Finnish type)	Gelsolin
40	NM_002128	HMGB1	High-mobility group box 1	HMG1
41	NM_001540	HSPB1	Heat shock 27kDa protein 1	HSP28/HSP27/Hsp25
42	NM_002166	ID2	Inhibitor of DNA binding 2, dominant negative helix-loop-helix protein	ID2
43	NM_000597	IGFBP2	Insulin-like growth factor binding protein 2, 36kDa	IGFBP-2
44	NM_000417	IL2RA	Interleukin 2 receptor, alpha	CD25
45	NM_000600	IL6	Interleukin 6 (interferon, beta 2)	IL-6
46	NM_000565	IL6R	Interleukin 6 receptor	IL-6 Ra
47	NM_002184	IL6ST	Interleukin 6 signal transducer (gp130, oncostatin M receptor)	GP130
48	NM_000210	ITGA6	Integrin, alpha 6	Integrin a6
49	NM_000213	ITGB4	Integrin, beta 4	Integrin b4
50	NM_002228	JUN	V-jun sarcoma virus 17 oncogene homolog (avian)	V-jun

Appendix. Continued.

Position	GeneBank	Symbol	Description	Gene name
51	NM_000222	KIT	V-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog	KIT
52	NM_001730	KLF5	Kruppel-like factor 5 (intestinal)	GC Box BP
53	NM_012427	KLK5	Kallikrein 5	KLK5
54	NM_000224	KRT18	Keratin 18	KRT18
55	NM_002276	KRT19	Keratin 19	Keratin 19
56	NM_002284	KRTHB6	Keratin, hair, basic, 6 (monilethrix)	KRTHB6
57	NM_023009	MLP	MARCKS-like protein	MacMarcks
58	NM_005043	MAP2K7	Mitogen-activated protein kinase kinase 7	JNKK2/MKK7
59	NM_002417	MKI67	Antigen identified by monoclonal antibody Ki-67	Ki67 (MKI67)
60	NM_005954	MT3	Metallothionein 3 (growth inhibitory factor (neurotrophic))	MT3
61	NM_182741	MUC1	Mucin 1, transmembrane	Episialin
62	NM_006166	NFYB	Nuclear transcription factor Y, beta	NFYB
63	NM_002506	NGFB	Nerve growth factor, beta polypeptide	NGF
64	NM_002507	NGFR	Nerve growth factor receptor (TNFR superfamily, member 16)	NGFR
65	NM_000269	NME1	Non-metastatic cells 1, protein (NM23A) expressed in	NM23
66	NM_002581	PAPPA	Pregnancy-associated plasma protein A	PAPPA
67	NM_000926	PGR	Progesterone receptor	PR
68	NM_002658	PLAU	Plasminogen activator, urokinase	uPA
69	NM_014330	PPP1R15A	Protein phosphatase 1, regulatory (inhibitor) subunit 15A	GADD34
70	NM_000314	PTEN	Phosphatase and tensin homolog (mutated in multiple advanced cancers 1)	PTEN
71	NM_000963	PTGS2	Prostaglandin-endoperoxide synthase 2 (prostaglandin G/H synthase and cyclooxygenase)	Cox-2
72	NM_002872	RAC2	Ras-related C3 botulinum toxin substrate 2 (rho family, small GTP binding protein Rac2)	Rac2
73	NM_000988	RPL27	Ribosomal protein L27	RPL27
74	NM_005978	S100A2	S100 calcium binding protein A2	CaN19
75	NM_006551	SCGB1D2	Secretoglobulin, family 1D, member 2	Lipophilin B
76	NM_002407	SCGB2A1	Secretoglobulin, family 2A, member 1	C3/Lipophilin
77	NM_002411	SCGB2A2	Secretoglobulin, family 2A, member 2	SCGB2A2
78	NM_001085	SERPINA3	Serine (or cysteine) proteinase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 3	AACT
79	NM_002639	SERPINB5	Serine (or cysteine) proteinase inhibitor, clade B (ovalbumin), member 5	Maspin
80	NM_000602	SERPINE1	Serine (or cysteine) proteinase inhibitor, clade E (nexin, plasminogen activator inhibitor type 1), member 1	PAI-1
81	NM_003486	SLC7A5	Solute carrier family 7 (cationic amino acid transporter, y+ system), member 5	CD98
82	NM_003125	SPRR1B	Small proline-rich protein 1B (cornifin)	SPRR1B
83	NM_003714	STC2	Stanniocalcin 2	STC2
84	NM_003225	TFF1	Trefoil factor 1 (breast cancer, estrogen-inducible sequence expressed in)	pS2
85	NM_003226	TFF3	Trefoil factor 3 (intestinal)	TFF3
86	NM_003236	TGFA	Transforming growth factor, alpha	TGF-a
87	NM_003246	THBS1	Thrombospondin 1	TSP1
88	NM_003247	THBS2	Thrombospondin 2	Thrombospondin2
89	NM_003248	THBS4	Thrombospondin 4	THBS4
90	NM_005424	TIE	Tyrosine kinase with immunoglobulin and epidermal growth factor homology domains	Tie-1
91	NM_006291	TNFAIP2	Tumor necrosis factor, alpha-induced protein 2	B94
92	NM_000043	TNFRSF6	Tumor necrosis factor receptor superfamily, member 6	Fas/Apo-1/CD95
93	NM_000639	TNFSF6	Tumor necrosis factor (ligand) superfamily, member 6	Fas ligand
94	NM_001067	TOP2A	Topoisomerase (DNA) II alpha 170kDa	TOP2 alpha
95	NM_000546	TP53	Tumor protein p53 (Li-Fraumeni syndrome)	p53
96	NM_003376	VEGF	Vascular endothelial growth factor	VEGF
97	L08752	PUC18	PUC18 Plasmid DNA	pUC18
98	L08752	PUC18	PUC18 Plasmid DNA	pUC18
99	L08752	PUC18	PUC18 Plasmid DNA	pUC18
100	—	Blank	—	—
101	—	Blank	—	—
102	—	Blank	—	—

Appendix. Continued.

Position	GeneBank	Symbol	Description	Gene name
103	NM_002046	GAPD	Glyceraldehyde-3-phosphate dehydrogenase	GAPDH
104	NM_002046	GAPD	Glyceraldehyde-3-phosphate dehydrogenase	GAPDH
105	NM_021130	PPIA	Homo sapiens peptidylprolyl isomerase A (cyclophilin A) (PPIA)	Cyclophilin A
106	NM_021130	PPIA	Homo sapiens peptidylprolyl isomerase A (cyclophilin A) (PPIA)	Cyclophilin A
107	NM_021130	PPIA	Homo sapiens peptidylprolyl isomerase A (cyclophilin A) (PPIA)	Cyclophilin A
108	NM_021130	PPIA	Homo sapiens peptidylprolyl isomerase A (cyclophilin A) (PPIA)	Cyclophilin A
109	NM_012423	RPL13A	Ribosomal protein L13a	RPL13A
110	NM_012423	RPL13A	Ribosomal protein L13a	RPL13A
111	NM_001101	ACTB	Actin, beta	b-actin
112	NM_001101	ACTB	Actin, beta	b-actin