Housekeeping genes in cancer: normalization of array data

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Biological maintenance of cells under variable conditions should affect gene expression of only certain genes while leaving the rest unchanged. The latter, termed "housekeeping genes," by definition must reflect no change in their expression levels during cell development, treatment, or disease state anomalies. However, deviations from this rule have been observed. Using DNA microarray technology, we report here variations in expression levels of certain housekeeping genes in prostate cancer and a colorectal cancer gene therapy model system. To highlight, differential expression was observed for ribosomal protein genes in the prostate cancer cells and β-actin in treated colorectal cells. High-throughput differential gene expression analysis via microarray technology and quantitative PCR has become a common platform for classifying variations in similar types of cancers, response to chemotherapy, identifying disease markers, etc. Therefore, normalization of the system based on housekeeping genes, such as those reported here in cancer, must be approached with caution.

INTRODUCTION

Microarray technology has revolutionized the era of functional genomics by enabling a global screening of differential gene expression between comparative biological samples. It has provided the momentum to keep pace with the rapidly emerging sequence information from the human genome project. The technology has evolved from the basic probe-to-target, gene-by-gene hybridization on a Northern blot (1,2) and from a grid screening of cDNA libraries (3) to multiple probe to multiple target hybridizations of up to two comparative samples for tens of thousands of genes (4). This rapidly evolving technology has proved to be a powerful tool in global gene expression analyses in various organisms (5,6), drug target validation (7), and identification of disease-specific genes and diagnostics (8). Recent advances in cancer research using microarrays have been made by the identification of distinct forms of large B-cell lymphomas (9) and by the distinction between acute myeloid leukemia and acute lymphoblastic leukemia, with a direct impact on therapy (10).

Normalization of microarray data involves standardizing the data against a set of reference points between the two comparative mRNA populations. One method to perform this normalization in any differential expression methodology is to use housekeeping genes as reference standards. Housekeeping genes, also termed "maintenance genes," by definition maintain the basic metabolic functions of the cell and provide support through the cell cycle and, thus, are expected to remain unchanged in their expression levels through various tissues or cells. However, with increasing expression studies using high-throughput technologies, the general concept of the constant expression of housekeeping genes is controversial, and their use as references is being approached with caution. Although a set of hundreds of these genes has been designated to be continually expressed through human development (11), the reliability of these genes as internal standards in gene expression experiments, following differential treatments or during diseased states, is precarious. One such example is cancer, in which the expression of some housekeeping genes has deviated from the norm. Overexpression of ribosomal proteins has been reported in certain cancers: colorectal (12), liver (13), and breast (14). Recently, overexpression of ribosomal proteins L7a and L37 has been reported in prostate cancer tissues when compared to a normal prostate epithelial cell line (15). An examination of the expression of 15 different housekeeping genes in colon cancer demonstrated substantial changes, particularly in those coding for metabolic enzymes (16). Interestingly, this study found little difference in ribosomal proteins. In sharp contrast, examination of melanocytic lesions showed minimal variation between nevi and melanoma (17). Housekeeping gene variation has also been observed in normal, primary cell cultures. Savonet et al. (18) examined the expression of three common housekeeping genes (glyceraldehyde-3-phosphate-dehydrogenase, β-actin, and cyclophilin) in primary cultures of normal thyrocytes under different mitogenic stimulations using Northern blot analysis. As in the previous examples, the vast majority of published observations addressing housekeeping gene-based normalization consist of single gene or small gene sets offering limited usefulness outside of the model system under study (19–23).

Normalization issues for large-scale gene expression studies performed on microarrays have, for the most part, moved away from simple comparison to a set of housekeeping genes to more complex statistical analyses that account for overall expression levels of all genes (24–29). Although these methods are quite effective when dealing with extremely large data sets, laboratories examining a small set of genes by microarray hybridization or alternative technologies (e.g., real-time PCR, Northern blot analysis, etc.) are left with little guidance in selecting appropriate reference genes.

To illustrate this dilemma, we have performed differential gene expression studies using microarray technology on three model systems: the prostate cancer cell lines, LNCaP and PC3, a colorectal cancer gene therapy model, and a phorbol ester [phorbol-12-myristate-13-acetate (PMA)]-

Table 1. Samples, Labeling Methodology, and Microarrays Used for These Experiments

Sample Name and Label		Labeling Method		Microarray Hybridized	
Cy3	Cy5	Direct	TSA	2400 Gene Array	Cancer Array
PC3	LNCaP	X	X	X	X
Jurkat	PMA-Stimulated Jurkat	X		X	X
DLD-1	Ad-MDA7-DLD-1	X			X

TSA, tyramide signal amplification; PMA, phorbol-12-myristate-13-acetate.

stimulated Jurkat T-cell line model. In contrast to prior studies, we detail the expession levels of 42 common housekeeping genes. These data illustrate the need for the careful selection of appropriate housekeeping genes for smaller scale gene expression experiments.

MATERIALS AND METHODS

Cell Culture and RNA Isolation

Prostate cancer cell lines, LNCaP and PC3, and Jurkat cells were obtained from the ATCC (Rockville, MD, USA). All three cell lines were propagated in RPMI 1640 (Invitrogen, Carlsbad, CA, USA) with 10% fetal bovine serum (FBS). DLD-1, a colorectal cell line, and T47D, a ductal carcinoma of breast cell line, were also obtained from the ATCC (nos. CCL-221 and HTB-133, respectively). Both cell lines were propagated in Dulbecco's modified Eagle's medium (DMEM; Invitrogen) with 10% FBS. Jurkat cells (human leukemic T-cell line) were stimulated with PMA at 50 ng/mL for 4 h. Unstimulated Jurkat cells were used as controls for comparison of differential gene expression. Total RNA was isolated using TRIZOL® (Invitrogen) and RNeasy® RNA isolation kit columns (Qiagen, Valencia, CA, USA), as described by the manufacturers and by a combined protocol as described in the MICROMAX™ Human cDNA System I-Direct manual (PerkinElmer Life Sciences, Boston, MA, USA).

Adenoviral Vector Transduction

Adenovirus-MDA7 (Ad-MDA7) was constructed at Introgen Therapeutics (Houston, TX, USA). Adenovirus-luciferase (Ad-luc) was also constructed at Introgen Therapeutics and was used in control vector experiments (data not shown). Cancer cells were plated at 5×10^5 per well in a 6-well format; 24 h later, the cells were infected at a multiplicity of infection (MOI) of 1000 viral particles per cell for 3 h. After infection, the cells were

washed with DMEM and further incubated in DMEM at 37°C in a 10% CO_2 incubator (Steri-Cult 200; Forma Scientific, Woburn, MA, USA). At different time intervals [24, 48, 72, and 96 h post-infection (p.i.)], the cells were trypsinized and lysed with sample buffer used for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The lysate was vortex mixed briefly, boiled at 100°C for 2 min, centrifuged (at 15,300× *g* for 2 min), run on SDS-PAGE, and Western blotted. The blot was probed for β-actin monoclonal antibody (Sigma, St. Louis, MO, USA).

Differential Gene Expression-Microarray Analysis

The following systems were used to perform the differential gene expression analysis: (*i*) the MICROMAX Human cDNA System I, containing 2400 gene screening microarrays, and (*ii*) the MICROMAX Direct System: Human Oncogenes and Tumor Suppressor Genes, containing 281 gene microarrays (both from PerkinElmer Life Sciences). Both microarray systems share the same common set of 42 housekeeping genes; however, the Human Oncogenes and Tumor Suppressor Genes microarray only shares 145 additional genes in common with the System I microarray. Its remaining genes are specific for cancer-related functions. For both microarray systems, the Cy™5 and Cy3 direct incorporation protocol was used to obtain fluorescently labeled cDNA probes from 100 µg of total RNA from each of the comparative treated-versus-untreated or normal-versus-disease cell lines (Table 1). The mRNA population in the total RNA from LNCaP and PC3 cells were fluorescently labeled (LNCaP RNA was labeled with Cy5-dUTP and PC3 RNA was labeled with Cy3-dUTP) by reverse transcription according to the kit protocol. Labeled cDNAs from both cell types were mixed and co-hybridized to the MICROMAX human cDNA glass microarrays (2400 genes) and the Human Oncogenes and Tumor Suppressor gene microarrays (281 genes). Similarly, the mRNA population in the total RNA from PMA-stimulated (labeled with Cy5-dUTP) and unstimulated (labeled with Cy3-dUTP) Jurkat cells was labeled via cDNA synthesis and co-hybridized on the microarrays as described above. In a third set of differential gene expression analysis experiments, total RNA from Ad-MDA7-transduced colorectal cancer cell line, DLD-1, was labeled with Cy5-dUTP, and the comparative RNA from control vector-transduced cells was labeled with Cy3-dUTP. Respective pairs of labeled targets were co-hybridized on the human cancer gene microarray. Prior to hybridization, the probe, resuspended in 20 µL of hybridization buffer, was denatured at 90°C for 2 min, placed on ice, centrifuged briefly, and applied to the microarrays with a coverslip in place. Hybridizations were performed at 65°C overnight in the hybridization buffer provided in the MICROMAX kits. Following hybridization, stringency washes were performed as described in the kit protocol. In addition, an alternative labeling procedure was used on the LNCaP and PC3 samples (MICROMAX Human cDNA Microarray System 1.1-TSA™). The tyramide signal amplification (TSA) detection procedure (30,31), which offers 50–100 fold higher sensitivity, used biotin- and fluorescein-labeled probes made from 1 µg each of input LNCaP and PC3 total RNA, respectively. Hybridization and stringency washes were as described above. The TSA detection process was then performed as described in the kit protocol.

Scanning and Imaging

The arrays were scanned at 10 µm resolution using a ScanArray™ 5000 fluorescence laser scanner (PerkinElmer Life Sciences). The Cy3 (PC3 or controls) and Cy5 (LNCaP or treated samples) images were scanned separately, with balancing of the two channels based on the hybridized intensities of the Cy3- and Cy5-labeled spiked control RNA and global balancing. Ratios of Cy5 and Cy3 signal intensities reflected the expression levels of mRNA in the LNCaP or treated cells versus PC3 or control cells (i.e., significant: ≥2-fold change in the ratio). Therefore, ratios ≥2 represented up-regulated genes, and ratios ≤0.5 represented down-regulated genes.

Table 2. Expression Ratios of Housekeeping Genes

No.	Gene Name	GenBank® Accession Number	Model I Prostate Cancer Cell Lines: LNCaP vs. PC3 Mean Ratio	Model I 95% Confidence Interval	Model II PMA-Induced Jurkat Cells vs. Jurkat Cells Mean Ratio	Model II 95% Confidence Interval	Model III Ad-MDA7-DLD-1 vs. DLD-1 Cells Ratio[a]
1	NAD(H)-specific isocitrate DH a-su precursor	U07681	1.13	0.19	0.90	0.03	0.79
2	α-Tubulin	K00558	1.11	0.08	0.93	0.12	0.84
3	**Ribosomal protein L10**	L25899	**2.39**	0.52	1.14	0.07	1.05
4	Ribosomal protein S9	U14971	1.91	0.60	1.07	0.08	1.04
5	mRNA for ribosomal protein S11	X06617	1.93	0.64	1.43	0.14	1.41
6	E2k α-ketoglutarate DH complex	S72422	0.84	0.24	0.79	0.29	0.54
7	**mRNA for ribosomal protein L37**	D23661	**2.48**	0.83	1.52	0.32	1.27
8	Pyruvate DH E1-α subunit (PDHA1)	L13318	1.20	0.16	1.33	0.28	1.51
9	**Ribosomal protein L23a**	U37230	**4.09**	1.16	1.59	0.40	1.24
10	S3 ribosomal protein	S42658	1.62	0.33	1.36	0.25	1.20
11	Succinate DH iron-protein su (sdhB)	U17248	0.66	0.14	1.07	0.07	0.75
12	mRNA for cytosolic malate DH	D55654	0.94	0.20	1.29	0.30	0.74
13	**Phospholipase A2**	M86400	**0.50**	0.23	1.03	0.19	**0.35**
14	Fumarase precursor (FH) mRNA	U59309	0.69	0.17	0.90	0.07	0.53
15	**mRNA for β-actin**	AB004047	0.87	0.44	0.59	0.27	**0.26**
16	H2A.X mRNA encoding histone H2A.X	X14850	1.29	0.46	1.03	0.20	0.65
17	EF-1d gene encoding elongation factor-1-d	Z21507	1.11	0.25	1.02	0.09	1.42
18	Cytosolic aspartate aminotransferase	M37400	1.55	0.31	1.24	0.04	0.91
19	Clone 23600 cytochrome c oxidase subunit IV	U90915	1.94	0.52	1.50	0.22	1.56
20	**Chaperonin protein (Tcp20)**	L27706	1.05	0.14	0.97	0.11	**0.25**
21	Acidic ribosomal phosphoprotein P0	M17885	1.59	0.30	1.14	0.08	0.90
22	9G8 splicing factor	L22253	0.93	0.23	1.14	0.26	0.57
23	pre-mRNA splicing factor SRp75	L14076	0.88	0.12	0.95	0.25	0.74
24	**PMI1 mRNA, phosphomannose isomerase**	X76057	**3.18**	1.60	1.06	0.18	1.31
25	mRNA encoding phosphoglycerate kinase	V00572	1.60	0.25	1.22	0.18	0.68
26	RNA polymerase II	L37127	1.17	0.33	0.85	0.17	0.50
27	snRNP polypeptide B	J04564	1.00	0.33	1.05	0.32	0.60
28	**Ribosomal protein L7a (surf 3) large su**	M36072	**5.78**	1.30	1.15	0.07	0.63
29	**mRNA for ribosomal protein L32**	X03342	**2.54**	0.56	1.50	0.18	1.13
30	**mRNA for ribosomal protein L3**	X73460	**4.19**	0.72	1.27	0.24	0.74
31	**Phosphoglycerate mutase (PGAM-B)**	J04173	**2.66**	0.61	1.01	0.19	0.58
32	**mRNA ornithine decarboxylase antizyme**	D78361	**2.17**	0.69	1.18	0.11	1.09
33	**mRNA for lactate DH-A**	X02152	1.23	0.16	1.03	0.27	**0.47**

Table 2. (Continued)

No.	Gene Name	GenBank® Accession Number	Model I Prostate Cancer Cell Lines: LNCaP vs. PC3 Mean Ratio	Model I 95% Confidence Interval	Model II PMA-Induced Jurkat Cells vs. Jurkat Cells Mean Ratio	Model II 95% Confidence Interval	Model III Ad-MDA7-DLD-1 vs. DLD-1 Cells Ratio[a]
34	IMP dehydrogenase type 1 mRNA	J05272	1.24	0.29	0.82	0.15	0.53
35	mRNA for H^+-ATP synthase subunit b	X60221	1.55	0.19	1.33	0.37	0.55
36	mRNA for eukaryotic initiation factor 4AI	D13748	1.89	0.44	1.23	0.01	0.50
37	Ubiquitin	M26880	1.38	0.32	1.45	0.07	1.19
38	Succinate DH flavoprotein subunit (SDH)	L21936	0.98	0.28	0.69	0.26	1.07
39	**Glyceraldehyde-3-phosphate DH**	M33197	**2.29**	0.38	1.27	0.13	0.69
40	**Aldolase A**	M11560	1.77	0.30	1.15	0.14	**0.42**
41	**mRNA for 23 kDa highly basic protein**	X56932	**2.78**	0.51	1.43	0.38	0.80
42	**mRNA encoding IMP**	V00530	**2.12**	0.62	1.25	0.42	0.99

Model I, prostate cancer model (LNCaP vs. PC3); Model II, PMA-stimulation Jurkat cells model; Model III, colorectal cancer (DLD-1 cells) gene therapy model. The Cy5/Cy3 expression ratios in red represent up-regulation, and those in green represent down-regulation (>2-fold change in both cases). The mean of the ratios and 95% confidence intervals were calculated using six replicates for Model I and three replicates for Model II. [a]Model III was analyzed only once; therefore, no mean ratio or 95% confidence interval is presented in the table. PMA, phorbol-12-myristate-13-acetate.

RESULTS AND DISCUSSION

Using comparative cancer models, we report the variation in the expression of certain housekeeping genes, which reflects their unreliability as global candidates for the normalization of differential gene expression analysis, particularly in microarray analysis-generating data for large populations of genes.

The background of this study was to investigate changes in gene expression in different cancer models. We have used one large set of 2400 human genes spotted on the MICROMAX general screening microarray, and another relatively small but more focused specific gene family (oncogenes and tumor suppressors) comprising 281 genes available as MICROMAX cancer microarray. Differential gene expression in the androgen-sensitive prostate cancer cell line, LNCaP, was compared with the androgen-insensitive cell line, PC3. LNCaP and PC3 represent the androgen-responsive and less invasive, and the androgen-insensitive and malignant cell lines of prostate cancer, respectively (32).

Recently, using subtractive hybridization of transcripts, Vaarala et al. (15) have identified the overexpression of several genes encoding ribosomal proteins in prostate cancer cell lines when compared to normal prostate cell line or hyperplastic-prostate tissue (HPL). Specifically, they have confirmed high levels of L7a and L37 transcripts. Interestingly, in our microarray analysis using Perkin-Elmer MICROMAX systems on LNCaP versus PC3, we have observed the overexpression of most of the ribosomal protein genes in LNCaP cells. These genes were a subset of a group of housekeeping genes (Table 2). Ribosomal proteins L10, L37, L23a, L7a, L32, and L3 were all found to be up-regulated in LNCaP cells compared to PC3 (Table 2; Figure 1A). In addition, six other housekeeping genes were also up-regulated in the LNCaP cells (Table 2). On the other hand, to compare the differential trend of this specific set of housekeeping genes (ribosomal proteins), microarray analyses performed on two other unrelated model systems were evaluated; one, a Jurkat cell PMA-stimulation model, and the other, a colorectal cancer gene therapy model. Array analyses on

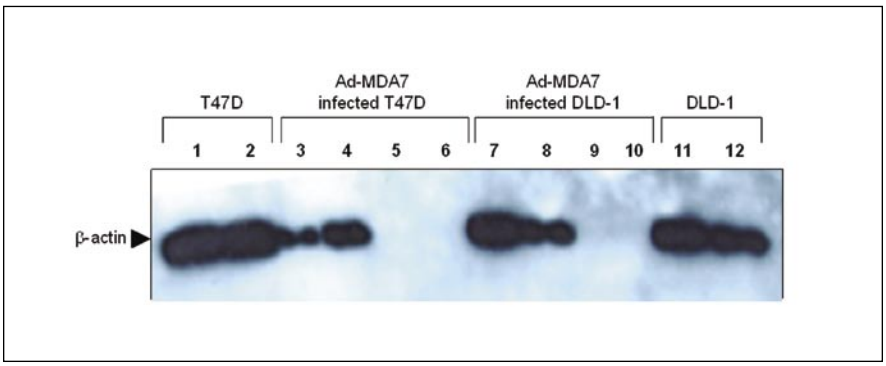

Figure 1. Loss of β-actin expression following MDA7-transduced breast cancer and colorectal cancer cell lines, as probed by Western blot analysis using monoclonal antibody for β-actin. Breast (T47D) and colorectal (DLD-1) cancer cell lines were transduced with Ad-MDA7. β-Actin expression was followed for 24, 48, 72, and 96 h post-infection (p.i.) using Western blot analysis and compared to its expression in untransduced cells. Lanes 1 and 2, the expression of β-actin in untransduced T47D cells (T47D control) probed at 24 and 96 h mock p.i., respectively. Lanes 3–6, the expression of β-actin in Ad-MDA7-infected T47D cells at 24, 48, 72, and 96 h, respectively. Lanes 7–10, expression of β-actin in Ad-MDA7-infected DLD-1 cells at 24, 48, 72, and 96 h, respectively. In both cases, note the decline in the expression of β-actin at 24 and 48 h p.i. and the complete disappearance of β-actin expression at 72 and 96 h. Lanes 11 and 12, expression of β-actin in untransduced DLD-1 cells (DLD-1 control) probed at 24 and 96 h mock p.i., respectively.

the latter two models clearly showed identical ribosomal protein genes exhibiting expression ratios within the expected range of 0.5–2.0, and thus, behaved as housekeeping genes (Table 2, Models II and III). Strikingly, in the colorectal cancer (DLD-1 cells) MDA7 gene therapy model, the microarray data suggest an almost 4-fold decline in the levels of β-actin in the MDA7-transduced cells (Table 2, Model III). Furthermore, in the MDA7-transduced breast (T47D) and colorectal (DLD-1) cancer cell lines, Western blot analysis confirmed this attenuation of β-actin expression. Decreased protein levels were observed at 24–48 h post-transduction, with complete suppression at 72–96 h (Figure 1). Untransduced cancer cells displayed constant β-actin expression (Figure 1, lanes 1 and 2, and lanes 11 and 12, respectively). Tumor suppressor genes, such as MDA7, are able to induce apoptosis and dismantle the cytoskeletal make-up of cancer cells (33). This is consistent with our findings from microarray and Western blot data. In a series of other studies done with normal cells (data not shown), Ad-MDA7 did not initiate killing nor apoptosis nor β-actin decomposition.

The overexpression of ribosomal proteins has been reported in several types of cancers (12–14), including prostate cancer (15). It has also been proposed that the elevated levels of certain ribosomal proteins in prostate cancer cell lines, such as LNCaP, may relate to their androgen sensitivity and insensitivity (15), and strikingly, ribosomal protein mRNAs, L7a and L37, have been reported to also serve as markers in prostate cancer tissues. Besides the differential expression of ribosomal proteins during different stages of prostate cancer as reported here and elsewhere (15), there is also growing evidence of the correlation between the differential expression of ribosomal proteins between different types of cancer. For example, the overexpression of the ribosomal protein L12 gene was reported in a prostate versus melanoma study (34). In another recent report, the ribosomal protein S6 kinase gene was found to be the most up-regulated and amplified in breast cancer and has been proposed as a potential target for treatment (35).

Overall, our data on three different cancer models analyzed using microarray gene expression profiling and Western blot analysis clearly indicate the differential expression of housekeeping genes. Routinely, housekeeping genes are and could be used as standards for normalization during slide scanning and data processing in microarray experiments. Various methods of normalization have been proposed and used (24–29). For instance, it is a recommended practice to perform a global balancing of the control and test samples based on the entire set of genes on the array in addition to the balancing of housekeeping genes. The global approach is less sensitive to the expression of individual genes and is often a preferred balancing method. Commonly used data normalization algorithms are also described in the documentation of commercially available software packages such as GenePix® (Axon Instruments, Foster City, CA, USA) and ImaGene™ (BioDiscovery, Los Angeles, CA, USA) .

In conclusion, from our observations and earlier reports, we suggest that dependence on housekeeping genes as a set of references should be approached with caution, particularly with aggressive use of expression-based technologies in the characterization of cancer and other diseases. Rather than blindly using a set of designated housekeeping genes as a reference for normalization, a detailed preliminary examination of the model system using either a large-scale microarray normalized globally or multiple single gene expression experiments analyzing a number of housekeeping genes (e.g., RNase protection assay, quantitative PCR, Northern blot analysis, etc.) should be performed to identify the appropriate controls.

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COMPETING INTERESTS STATEMENT

The authors declare no competing interests.

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