Methodology article

Sensitivity and fidelity of DNA microarray improved with integration of Amplified Differential Gene Expression (ADGE)

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Abstract

Background: The ADGE technique is a method designed to magnify the ratios of gene expression before detection. It improves the detection sensitivity to small change of gene expression and requires small amount of starting material. However, the throughput of ADGE is low. We integrated ADGE with DNA microarray (ADGE microarray) and compared it with regular microarray.

Results: When ADGE was integrated with DNA microarray, a quantitative relationship of a power function between detected and input ratios was found. Because of ratio magnification, ADGE microarray was better able to detect small changes in gene expression in a drug resistant model cell line system. The PCR amplification of templates and efficient labeling reduced the requirement of starting material to as little as 125 ng of total RNA for one slide hybridization and enhanced the signal intensity. Integration of ratio magnification, template amplification and efficient labeling in ADGE microarray reduced artifacts in microarray data and improved detection fidelity. The results of ADGE microarray were less variable and more reproducible than those of regular microarray. A gene expression profile generated with ADGE microarray characterized the drug resistant phenotype, particularly with reference to glutathione, proliferation and kinase pathways.

Conclusion: ADGE microarray magnified the ratios of differential gene expression in a power function, improved the detection sensitivity and fidelity and reduced the requirement for starting material while maintaining high throughput. ADGE microarray generated a more informative expression pattern than regular microarray.

Background

Gene expression profiles generally present signatures for cells at specific states, homeostatic or stressed, providing fingerprints critical in identifying regulatory pathways. DNA microarray technologies are designed to reveal gene expression profiles by simultaneously detecting the expression levels on a genomic scale [1,2]. They are now used to profile gene expression in a variety of model systems, such as antioxidant response [3] and tumor staging [4]. However, the hybridization based approach suffers from limitations including, low sensitivity for genes with small changes of expression level, limited accuracy with potential for high experimental error [5] and necessity for a large amount of biological starting material. The

Amplified Differential Gene Expression (ADGE) technique was designed to quadratically magnify the ratios of gene expression [6]. Briefly, the control and tester cDNA's are digested with *Taq* I restriction enzyme, then ligated with the CT and TT adapters, respectively. The adapter-linked control and tester DNA are reassociated through mixing at a ratio of 1:1, denaturing and annealing. The DNA reassociation results in the quadratic magnification of expression ratios for the up- and down-regulated genes in control and tester samples. The ADGE magnification is theoretically governed by the algebra formula (a + b)(a' + b') = aa' + bb' + a'b + ab' where aa' represents the control DNA, bb' represents the tester DNA and a'b and ab' represent hybrid DNA' s. For example, for a gene up-regulated 2-fold in tester over control, the formula is (a + 2b)(a' + 2b') = aa' + 4bb' + 2a'b + 2ab'. After DNA reassociation, the ratio of bb' /aa' increases from 2 to 4. If expression of another gene is down-regulated 3 times in the tester, the formula is (3a + b) (3a' + b') = 9aa' + bb' + 3a'b + 3ab'. Therefore, the ratio of aa' /bb' increases from 3 to 9 after reassociation. For a gene with a ratio of 1:1, the ratio is kept unchanged after reassociation. The reassociated DNA is amplified by using PCR with the CT primer complementary to the CT adapter or the TT primer complementary to the TT adapter. The CT primer amplifies the control DNA (aa') exponentially and hybrid DNA (a'b and ab') linearly while the TT primer amplifies the tester DNA (bb') exponentially and hybrid DNA linearly since hybrid DNA has two different adapters at the ends. The PCR not only exponentially amplifies the control and tester DNA but also separates them from each other. The expression patterns for over-expressed, repressed and unchanged genes were profiled with the ADGE technique [6]. However, the throughput of ADGE is low since it displays an average of 4 genes at a time with agarose gels. Our present paper combines the ADGE technique with DNA microarray (hereafter called ADGE microarray) in order to integrate the high throughput of DNA microarray with the ratio magnification and the PCR amplification of ADGE. The combination of ADGE and DNA microarray was used to analyze differential gene expression in a selected drug resistant cell line.

The prodrug TLK286 [γ-glutamyl-α-amino-β(2-ethyl-N,N,N,N-tetrakis(2-chloroethyl)phosphorodiamidate)-sulfonyl-propionyl-(R)-(-)phenylglycine] is activated by glutathione S-transferase (GST) P1-1 and to a lesser degree A1-1 and generates tetrakis chloroethyl alkylating moieties that can react with cellular nucleophiles. Cellular response to chronic TLK286 exposure included a two-fold decrease in GSTP1-1 protein levels, confirming a GSTP1-1 mediated mechanism of activation [7]. Mouse embryo fibroblast cells from GSTP1-1 deficient animals elevated the expression of signal-regulated kinases ERK1/ERK2 and reduced the doubling time of cell proliferation [8]. That

paper also found that a clone of HL60 cells resistant to TLK199, an inhibitor of GSTP1-1, has been shown to have elevated activities of c-Jun NH_2-terminal kinase (JNK) and ERK1/ERK2. In addition, GSTP1-1 acts as a negative regulator of JNK primarily through direct protein:protein interactions [9,10]. These data suggest that GSTP1-1 has a role in regulation of kinase activities and cell proliferation [8,11–13]. Because of this available background information, the HL60 wild type and TLK286 resistant cell lines were chosen as model systems for analysis by ADGE microarray. The results show that ADGE microarray improves the sensitivity and fidelity of detection and reduces the requirement for starting material while maintaining the high throughput of regular microarray. The gene expression profile for the resistant cells revealed with ADGE microarray reflected changes in the expression of GSTP1-1 and genes related to kinase and proliferation pathways.

Results

ADGE microarray magnified ratios
The ratios detected with ADGE microarray were greater than the corresponding input ratios. The input ratios of 2, 3, 4 were detected as averages of 3.6, 5.3 and 9.1, respectively, after normalization with the ratio of 1. In contrast, these samples were detected as 1.5, 2.0 and 2.7 with regular microarray, values that were less than their corresponding input ratios (Fig. 1). The power regression best fits the data trend of ADGE microarray with the largest R^2 of 0.97 among linear, logarithmic, polynomial, power and exponential. The relationship between the detected ratio (y) and the input ratio (x) for ADGE microarray is $y = 1.05x^{1.55}$ (Fig. 2A). This relationship is close to the theoretical one of quadratic magnification $y = x^2$. Therefore, the ratios were magnified in power function within this low range. However, the linear regression best fits the data trend of regular microarray with the largest R^2 of 0.96. The relationship for regular microarray is $y = 0.56x + 0.39$, which suggests that the ratio detected with regular microarray represents about half the input ratio within this range.

The magnification of power function continued until the 6-fold input ratio. After that, the magnitude of ADGE magnification leveled off (Fig. 2B). The overall relationship between detected ratios and input ratios had a logarithmic trend: $y = 15.99\ln(x) - 6.14$ with $R^2 = 0.84$.

ADGE microarray improved detection sensitivity
The ratio magnification was also observed when the ADGE microarray method was applied to the whole microarray chip. The MA plot of ADGE microarray has wider upward and downward distribution from the central area than that of regular microarray, particularly for the spots with high intensity (Fig. 3A and 3B). 578 of
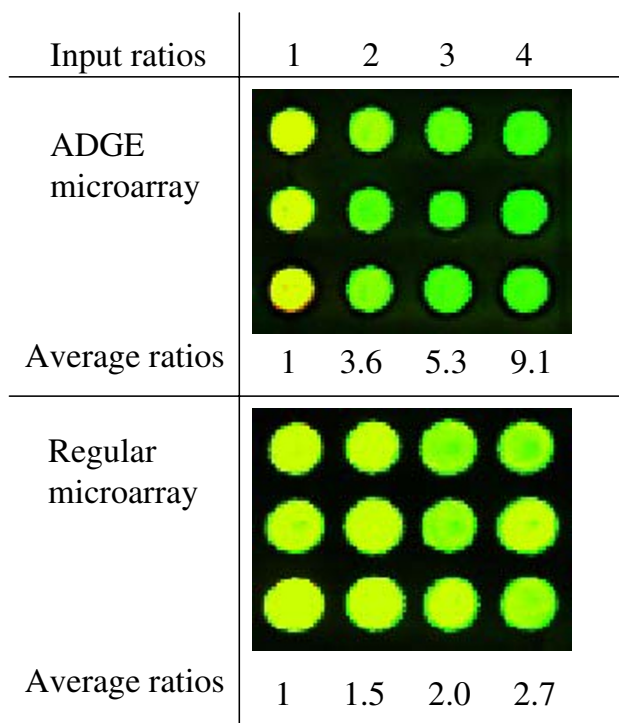
Figure 1
Composite images of ADGE microarray and regular microarray. The clones corresponding to the contiguous area of twelve spots (3 × 4) were amplified by using PCR with the primers having a *Taq I* site at the end. After cut with *Taq I*, the same amount of DNA for each clone was ligated to the CT and TT adapters. The CT and TT adapter-linked DNA fragments were mixed in ratios of 1:1 for the three clones of the first column, 1:2 for the clones of the second column, 1:3 for the clones of the third column, 1:4 for the clones of the fourth column. The top panel is the result of ADGE microarray while the bottom panel is the result of regular microarray. The ratios are represented by Cy5 (green) to Cy3 (red) and normalized with the value of clones in the first column. The detected ratios are averages of three spots in each column.

10,000 genes were detected at 2–4 fold up-regulated and 460 genes at 2–4 fold down-regulated with ADGE microarray (Fig. 3D); 107 and 38 genes detected at >4 fold up- and down-regulated, respectively. In contrast, with regular microarray, 227 and 244 of 10,000 genes were detected at 2–4 fold up- and down-regulated, respectively; 10 and 30

genes at >4 fold up- and down-regulated, respectively. Most outliers in regular microarray were the results of spontaneous experimental error, with low spot intensities. When only these genes with >99% confidence level of t test are considered, one and three genes were detected at 2–4 fold up- and down-regulated, respectively, with regular microarray; one gene detected at >4 fold down-regulated (Fig. 3D). However, with ADGE microarray, there were 63 genes >4 fold up-regulated, 220 genes 2–4 fold up-regulated, 92 genes 2–4 fold down-regulated and 14 genes >4 down-regulated. The ADGE magnification raised small changes in gene expression to a level beyond the inherent limit of DNA microarray and improved detection sensitivity.

ADGE microarray improved detection fidelity

Multiple steps were integrated to improve the fidelity of detection in ADGE microarray. The ADGE magnification increased the magnitude of the expression ratios beyond the detection error of standard microarray. PCR amplification of DNA templates dramatically increased the amount of probe. The CT and TT adapters at the DNA fragment ends facilitated efficient labeling of Cy3 and Cy5, thus enhancing signal intensity. Variances of Cy3 and Cy5 intensities were less in ADGE microarray than in regular microarray (Fig. 4A and 4B). 6888 and 6663 of 10,000 genes in ADGE microarray had variances of < 0.5 in Cy3 and Cy5 channels, respectively, while in regular microarray, there are only 717 and 741 such genes, suggesting that the results among replicates were more consistent with less variation in ADGE microarray.

The confidence levels of t tests reflect the magnitude of consistent differences between HL60 and HL60/TLK286 among replicates. 836 of 10,000 genes were identified at 99% confidence level, 2013 genes at 95–98%, 753 genes at 90–94% with ADGE microarray. In contrast, for regular microarray, 85 genes were detected at 99%, 367 genes at 95–98% and 409 genes at 90–94% (Fig. 4C).

Among the genes with >99% confidence levels and >4 fold changes, five genes were found duplicated on the chip. The results of the duplicated spots were similar (Table 1). For example, bmi-1 was detected at 7.96 and 7.29 in both spots.

ADGE microarray revealed genes associated with drug resistance

ADGE microarray was used to compare expression patterns for the TLK286 resistant cell line and its wild type counterpart by screening the expression pattern of 20,000 genes. Ninety three genes were selected with the threshold average ratio >4.0 and confidence level >99%. Among the 93 genes, 12 had functions related to cell proliferation and kinase pathways (Table 2). In addition, expression of
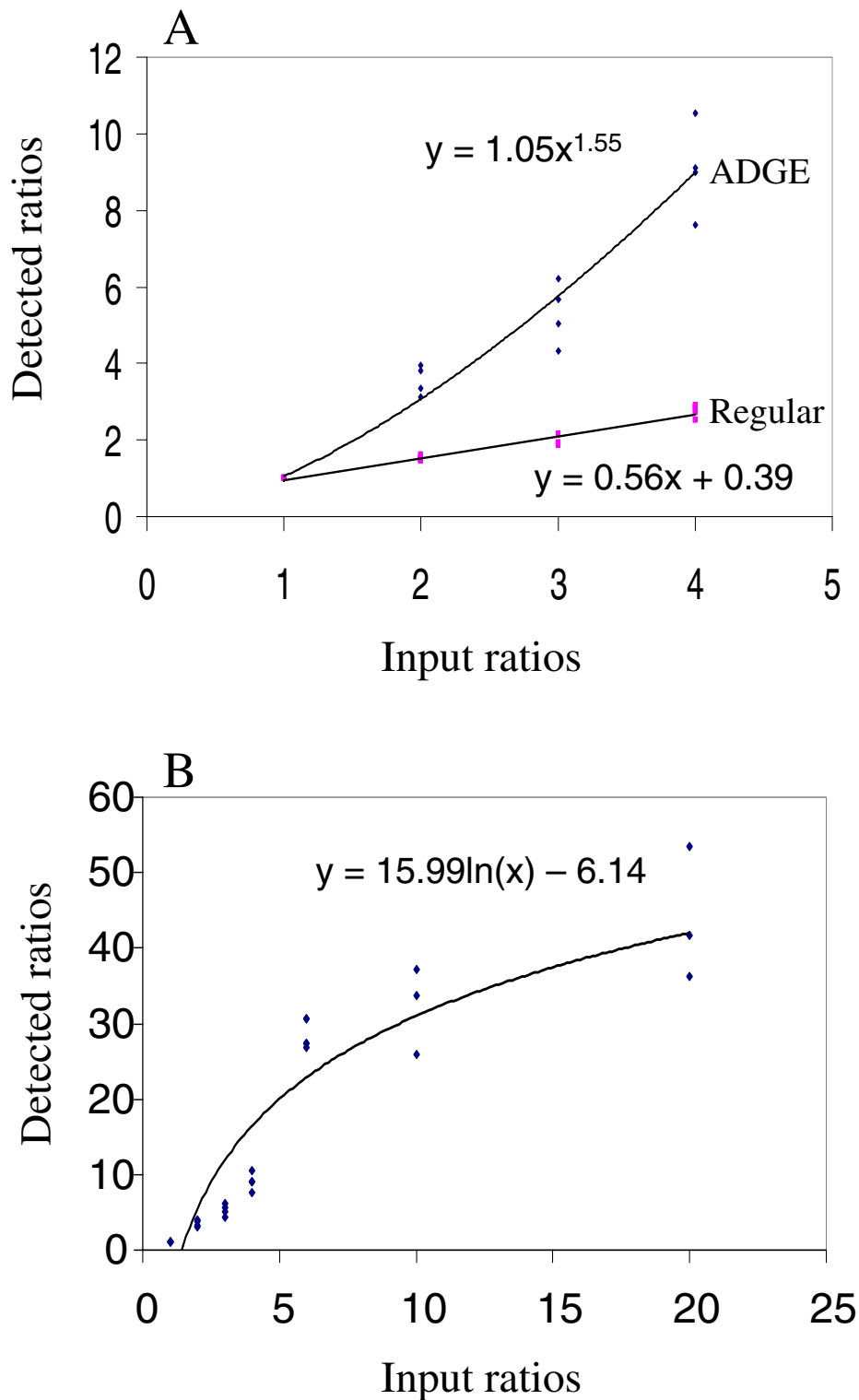
Figure 2
Relationship between detected ratios (y) and input ratios (x). The relationship is $y = 1.05x^{1.55}$ with $R^2 = 0.97$ for ADGE microarray while it is $y = 0.56x + 0.39$ with $R^2 = 0.96$ for regular microarray within the input ratio of 4 (panel A). Within the input ratio range of 1~20, the relationship is $y = 15.99\ln(x) - 6.14$ with $R^2 = 0.84$ for ADGE-microarray (panel B).
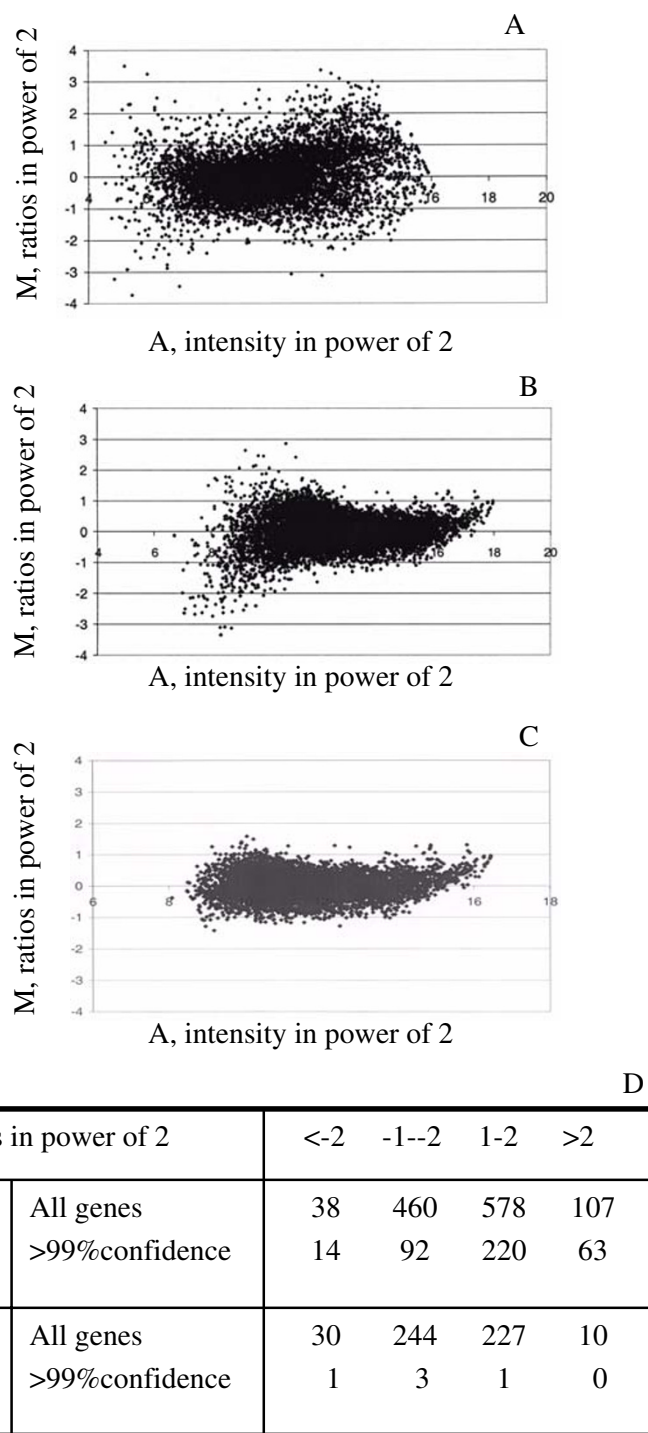
M, ratios in power of 2		<-2	-1--2	1-2	>2
ADGE	All genes	38	460	578	107
	>99%confidence	14	92	220	63
Regular	All genes	30	244	227	10
	>99%confidence	1	3	1	0

Figure 3
The MA plots of ADGE microarray and regular microarray. A is the average of $\log_2$Cy5 and $\log_2$Cy3, representing intensities of spots. M is the difference of $\log_2$Cy5 and $\log_2$Cy3, representing the expression ratios in the power of 2, with positive values for up-regulated genes, negative values for down-regulated genes and 0 for unchanged genes. Panel A: ADGE microarray with HL60 vs HL60/TLK286, average of three replicates. Panel B: regular microarray with HL60 vs HL60/TLK286, average of three replicates. Panel C: ADGE microarray with HL60 vs HL60, average of two replicates. Panel D: number of differential genes selected from all genes on the chip or from genes with > 99% confidence level.
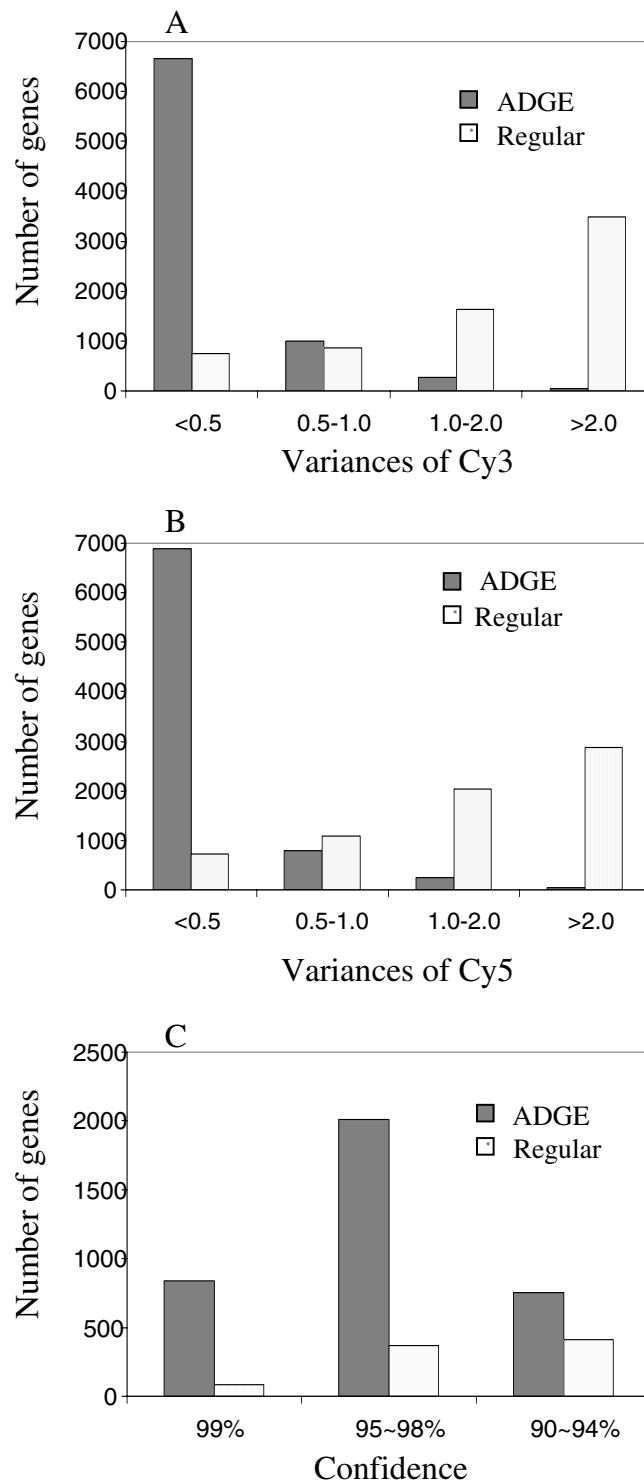
Figure 4
The variances of Cy3 (HL60) (panel A) and Cy5 (HL60/TLK286) (panel B) in ADGE microarray and regular microarray. The normalized values of Cy3 and Cy5 were used to calculate the variances. Panel C is the number of genes with confidence levels of 90% or greater in ADGE microarray and regular microarray. The confidence level is a result of t-test for each gene.

Table 1: Comparison of ADGE microarray results between two duplicated spots

Gene name	GenBank ID	Ratios[a]	std[b]	Confid.[c]
bmi-1	T87515	7.96	0.35	99.98
bmi-1	AA478036	7.29	2.53	99.06
Human clone 23933	H56918	6.92	0.75	99.88
Human clone 23933	H56918	6.00	0.90	99.76
MDS023 protein	N52373	5.76	1.60	99.08
MDS023 protein	N52373	5.66	1.41	99.23
protein phosphatase 2	AA599092	5.69	1.24	99.41
protein phosphatase 2	AA599092	5.43	0.21	99.98
acetylglucosaminyltransferase	AA485653	5.45	0.75	99.77
acetylglucosaminyltransferase	AA485653	4.23	0.94	99.15

a: average ratio of three replicates, represented by the values of HL60/TLK286 to HL60. b: standard deviation. c: confidence level of t test. (%)

Table 2: Expression of GSTP1 and genes related to proliferation and kinase pathways detected with ADGE microarray

Gene name	GenBank ID	Ratios	std	Confid.	RT-PCR	Known functions
nucleoporin 88 kD	AA488609	11.40	5.38	99.04	1.4	MAPK translocation
p21-activated kinase 1	AA890663	6.70	2.10	99.17	1.5	JNK MAP kinase pathway
spindlin	AA428181	4.84	0.92	99.56	1.4	MAP kinase pathway
dual specificity phosphatase 10	AA056608	4.05	0.30	99.90	1.2	dephosp. stress-act. kinases
protein phosphatase 2	AA599092	5.43	0.21	99.98	1.6	phosphatase
DC2 protein	AA001745	4.98	1.10	99.43	1.4	cell differentiation
M-phase phosphoprotein 1	AA425160	4.95	0.75	99.71	1.3	cells cycle
KIAA0008 gene product	W93717	4.90	0.52	99.85	1.2	cell cycle regulated
cullin 1	AA486790	4.56	0.67	99.67	1.6	ubiquitination of cyclin D1
tousled-like kinase 2	AA599008	4.40	0.55	99.77	1.0	cell cycle regulation
chrom. 7 open reading frame 2	W46783	4.38	0.88	99.31	1.4	differentiation-related
replication factor C	N93924	4.02	0.59	99.61	1.0	cell proliferating activator
GSTP1	R33642	-2.67	0.24	99.69	-1.5	catalytic and ligand binding

GSTP1-1 was reduced in the HL60/TLK286 cell line, which was consistent with a previous study [7]. Quantitative RT-PCR confirmed the results of ADGE microarray for eleven of thirteen genes. The other two genes were detected unchanged with RT-PCR. The gene expression profile revealed with ADGE microarray supported the principle of GSTP1-1 mediated regulation of cell proliferation and kinase pathways.

Discussion

The ADGE technique is a method designed to magnify the ratios of gene expression before detection. The ratio magnification was reported with a gel system [6] and was observed in this study with DNA microarray (Fig. 1 and 2). The magnification of a power function was found up to a 6-fold input ratio or 30-fold detected ratio. The magnitude of magnification leveled off after that. Since different dilutions, different cycles of PCR and dye reverse were used, ADGE magnification can be applied for up- and down-regulated genes with different abundance. One reason for the level-off might be the capacity of DNA microarray to detect large ratios. For instance, the maximum value of spot intensity is 65,000 in an Affymetrix scanner. If an abundant gene has an intensity of 1000 in another scanning channel, the largest detectable ratio will be 65. Another explanation might be the saturation of PCR for abundant genes. The regression equations of the detected values with input values not only demonstrate the magnitude of magnification with ADGE technology, but also provide a guideline to interpret the results of ADGE microarray. The quantitative relationship between input values (x) and detected values (y) was $y = 0.56x + 0.39$ for regular microarray. Thus, regular microarray may under-estimate differences of gene expression between two samples.

ADGE methodology not only magnifies the differential expression ratios but also amplifies the amount of templates with PCR. Exponential amplification by PCR dramatically increases the amount of probes and reduces the

requirement for starting material. Based on the current working protocol, 10 μg of total RNA from control and tester samples was used to generate 160 μl of reassociated DNA. 2 μl of the reassociated DNA was needed to make probe for one slide hybridization. Thus, 125 ng of total RNA can be used for one slide hybridization. Therefore, ADGE microarray could provide a platform for using 160–200 fold less total RNA than regular microarray (which generally requires 20 μg of total RNA). It is also comparable to other RNA amplification methods, such as antisense RNA amplification where 0.25 – 3.0 μg of total RNA is required [14] and Amine-modified random primer where 1.0 μg of total RNA is required [15]. 20–30 PCR cycles are recommended since too few cycles will reduce the difference between the exponentially amplified tester or control DNA and the linearly amplified hybrid DNA and too many cycles will saturate abundant genes.

The adapters at the DNA fragment ends facilitate efficient incorporation of Cy3 and Cy5 into DNA templates and enhance signal intensity. Both methods of direct and indirect labeling were used in this study. With direct labeling, Cy3-dCTP is incorporated into control and Cy5-dCTP into tester DNA during the PCR amplification of the hybridized DNA templates. The use of Cy dyes can be reversed if required. With indirect labeling, aminoallyl-dUTP is incorporated into control and tester DNA with PCR of 3 cycles after the reassociated DNA templates are amplified. The aminoallyl-dUTP labeled DNA templates are in turn coupled with Cy dyes. The use of aminoallyl-dUTP in PCR amplification of the hybridized DNA template is not recommended. It is also possible to adapt other methods of signal enhancement to the labeling procedure. For example, the 3DNA fluorescent dendrimer probes (Genisphere, Montvale, NJ) can be attached on the CT and TT primers which then are used to amplify the control and tester DNA's. Since both strands of the probe DNA are labeled with direct labeling or indirect labeling, the specific intensity is enhanced. The ratio of signal to background was improved in ADGE microarray. For example, on slides of 10,000 genes, 1301 genes were detected at ratios >18 and 7988 genes at ratios < 9 with ADGE microarray. In contrast, 578 genes were detected at ratios >18 and 8678 genes at ratios < 9 with regular microarray. The reason that overall spot intensities in Fig. 3A are lower than in Fig. 3B is that smaller gain values were used for ADGE microarray when scanning microarray chips.

The integration of these features in ADGE microarray improves detection sensitivity and fidelity. ADGE magnification raised small changes in gene expression to a level beyond the inherent limit of DNA microarray. Thus, genes with small expression changes could be identified more accurately and false negatives could be minimized with ADGE microarray. 77 genes with >99%

confidence level and >4 fold changes were identified with ADGE microarray, compared to only one such gene with regular microarray (Fig. 3D). Therefore, a more informative gene profile was revealed with ADGE microarray than with regular microarray. The probability that the ADGE magnification introduces false positives is low. The HL60 vs HL60 self-hybridization showed that the relationship of Cy3 (y) and Cy5 (x) is y = 1.02x - 0.21 with R^2 = 0.92; none of the 10,000 genes was detected at ≥ 4 fold change with confidence level ≥ 99% (Fig. 3C). Eleven of thirteen genes have consistent results between ADGE microarray and quantitative RT-PCR. The other two genes were detected unchanged with RT-PCR (Table 2).

Experimental artifacts in microarray data are intensity-dependent and tend to occur primarily for weak spots [16]. The nucleporin 88 kD (AA488609) with the largest standard deviation in the Table 2 had weak signal. Exponential amplification of DNA templates and double strand labeling in ADGE microarray dramatically enhances the signal intensity of spots. Combined with magnified ratios, the enhanced signal intensities improve the data quality and detection fidelity in ADGE microarray. More genes have low variances in both Cy3 and Cy5 channels and high confidence levels in ADGE microarray than in regular microarray (Fig. 4). The similarity and reproducibility of results between duplicate spots also suggests the improvement of detection fidelity (Table 1).

The sensitive and accurate method of ADGE microarray provides a novel tool to reveal informative gene profiles. 12 of the 93 differential genes were clustered in cell proliferation and kinase pathways. Expression changes of genes related to regulation of cell proliferation may be directly pertinent to the resistant phenotype or may be a consequence of cell growth under drug stressed conditions. Adaptive changes in kinase expression have been characterized in a variety of drug resistant cells. For example, the stress kinase JNK1 was activated during TLK286 induced apoptosis [17] and JNK is known to be activated during apoptosis induced by a variety of stress stimuli [18]. However, in HL60/TLK286 resistant cells, we observed an overexpression of two phosphatases *PP2* (AA599092) and *MKP5* (AA056608) involved in JNK dephosphorylation and inactivation (Table 2). PP2 is a serine/threonine phosphatase which dephosphorylates JNK during inflammatory cell signaling [19], while MKP5 is a member of the dual specificity phosphatase family which selectively dephosphorylates stress activated MAP kinases including JNK [20]. The overexpression of these two phosphatases might contribute in part to the resistance of HL60/TLK286 by impairing the activity of JNK. Kinase pathways, particularly those involving ERK and JNK, are suggested to have prominent roles in regulation of proliferation pathways in a variety of cell lines [11–13].

Reduced expression of *GSTP1-1* in the resistant cells provides a plausible link with the JNK and ERK regulation pathways in the resistant cells. Although the interaction among *GSTP1-1* and genes of proliferation and kinase pathways needs to be verified and the roles of other differential genes in the TLK286 resistant cell line need further investigation, the expression pattern generated with ADGE microarray provides a blueprint for designing further experiments.

Conclusions

ADGE microarray is the combination of ADGE technique and DNA microarray. It magnified the ratios of differential gene expression in power function, improved detection sensitivity and fidelity and reduced the requirement for starting material while maintaining high throughput. ADGE microarray generated a more informative expression pattern than regular microarray.

Methods

Cell lines

The HL60/TLK286 cell line is resistant to the prodrug TLK286 and derived from the wild-type HL60 by stepwise selection. Both HL60/TLK286 and HL60 cell lines were cultured in RPMI-1640 medium supplemented with 2 mM L-glutamine, 50 U/ml penicillin, 50 µg/ml streptomycin and 10% heat-inactivated fetal bovine serum. Cells were maintained in a humidified atmosphere of 5% CO_2 at 37°C.

Adapters and primers

The sequences of the adapters and primers were modified from reference [6] for better priming efficiency.

CT adapter: AAC TGC AGG AGG GAC AGT TGA AGG AGG CA CC TCC GTG C

TT adapter: AAC TCA GAG GTG AGA CAG GAG TGG AGG CA CC TCC GTG C

CT primer: GCA GGA GGG ACA GTT GAA GGA G

TT primer: CAG AGG TGA GAC AGG AGT GGA G

Verification of ratio magnification

In order to verify the ratio magnification for ADGE and to build a relationship between input and detected ratios, a contiguous area of twelve spots (3 × 4) on the microarray chip was selected. The corresponding clones were amplified by using PCR with the primers having a *Taq I* site at the end. The PCR products were then purified and cut with *Taq I* at 65°C for 2 hr. The same amount of *Taq I* fragments for each clone was ligated to the CT and TT adapters at 16°C overnight. The CT and TT adapter-linked DNA fragments were mixed in ratios of 1:1 for the three

clones of the first column, 1:2 and 1:6 for the clones of the second column, 1:3 and 1:10 for the clones of the third column, 1:4 and 1:20 for the clones of the fourth column (see clone arrangement in Fig. 1). Reverse ratios were also made for all except 1:1. A fraction of each mixture was reassociated by denaturing at 95°C for 5 min and annealing at 68°C for 20 hr. Due to high concentration of templates and possibility of effects of template abundance, the reassociated DNA was diluted 1000 or 100,000 times, then amplified using PCR with the CT or TT primers. A PCR reaction was set up with 1 µl of the reassociated DNA, 5 µl of 10x Clontech PCR buffer, 1 µl of 10 mM dNTPS, 2 µl of 10 µM CT primer or TT primer, 1 µl of Clontech cDNA polymerase and 40 µl of ddH$_2$O. The reaction cycling conditions were 72°C for 5 min (for filling in the adapter ends), 94°C for 1 min, then 15 or 20 cycles of 94°C for 30 s, 66°C for 30 s, 72°C for 90 s, then 72°C for a final extension. The PCR products were purified with Qiagen PCR Purification Kit (Qiagen, Valencia, CA) and eluted into 42 µl of ddH$_2$O, which was used for indirect labeling with PCR. The PCR labeling reaction was set up with 42 µl of DNA templates, 5 µl of 10x Clontech PCR buffer, 1 µl of 10 mM dNTPS containing 8 mM aminoallyl-dUTP and 2 mM dTTP, 1 µl of 10 µM CT primer or TT primer and 1 µl of Clontech cDNA polymerase. The reaction cycling conditions were 94°C for 1 min, then 3 cycles of 94°C for 30 s, 64°C for 30 s, 72°C for 90 s, then 72°C for a final extension. The PCR product was ethanol-precipitated, resuspended in 5 µl 2x coupling buffer, then coupled with 5 µl of Cy3 or Cy5 dye (Amersham Pharmacia, Piscataway, NJ) for 1 hr in the dark. The labeled DNA was mixed with 50 µl of 100 mM sodium acetate (pH 5.2), purified with Qiagen PCR Purification Kit, reduced to a final volume of 7.5 µl with a speed vac, mixed with 3.75 µl of 20x SSC, 0.75 µl of 10% SDS, 1.5 µl of 1 µg/µl salmon DNA and 1.5 µl of 50x Denhardt's solution, denatured at 95°C for 5 min, cooled on ice and incubated at 42°C for 15 min. The denatured Cy3 and Cy5 DNA were mixed and loaded onto the area of the twelve selected spots on the microarray chip. The hybridization and washing conditions were the same as for regular microarray.

As a control, another fraction of the mixture of each ratio from 1 to 4 directly proceeded to the PCR indirect labeling without DNA reassociation. The conditions for chip hybridization and washing were the same as above. Four replicates were performed for both the control and the combination of ADGE and microarray.

Regular DNA microarray

Total RNA was isolated from both HL60 and HL60/TLK286 cell lines with a Qiagen RNeasy Midi Kit. Regular microarray was carried out following the manufacturer's instructions for the FairPlay™ microarray labeling kit

(Stratagene, La Jolla, CA). Briefly, twenty μg of total RNA from HL60 or HL60/TLK286 cells was reverse-transcribed into single stranded cDNA. The cDNA was purified with ethanol precipitation and resuspended in 5 μl of 2x coupling buffer, then coupled with 5 μl of Cy3 or Cy5 dye for 1 hr in the dark. The labeled HL60 cDNA and HL60/TLK286 cDNA were combined and purified. The labeled cDNA was mixed with 1.5 μl of 10 μg/μl Cot-1 DNA, 1.5 μl of 8 μg/μl poly d(A), 1.5 μl of 4 μg/μl yeast tRNA, 4.5 μl of 20x SSC and 0.75 μl of 10% SDS, heated at 99°C for 2 minutes, and then incubated at 45°C for 15 minutes. The labeled DNA was loaded onto a microarray chip. A hybridization chamber was assembled with the microarray chip and submerged in a water bath at 60°C overnight. The microarray chip was washed in wash buffer I (2x SSC, 0.1% SDS) for 5 minutes, then in wash buffer II (1x SSC) for 5 minutes and wash buffer III (0.2x SSC) for 5 minutes. The slide was dried by centrifuging at 650 rpm for 5 minutes and scanned with Affymetrix 428 Array Scanner using the Cy3 and Cy5 channels. Three replicates were performed on the first set of human microarray chips containing 10,368 genes each made at the Fox Chase Cancer Center Microarray Facility.

ADGE microarray

The protocol of ADGE microarray is available on http://www.fccc.edu/research/ADGEmicroarray. Briefly, ten μg of total RNA was reverse-transcribed into single stranded cDNA with oligo(dT)$_{12-18}$. Then the double stranded cDNA for HL60 and HL60/TLK286 was generated with the cDNA Synthesis System (Life Technologies, Rockville, MD). After phenol extraction and ethanol precipitation, the cDNA was resuspended in 25 μl of ddH$_2$O. Both HL60 and HL60/TLK286 cDNA were digested with 3 μl (30 units) of the restriction enzyme *Taq* I in a final volume of 30 μl. The *Taq* I fragments of HL60 cDNA were ligated with the CT adapter at 16°C overnight with 3 μl (9 units) of T4 ligase (Promega, Madison, WI) while the *Taq* I fragments of HL60/TLK286 cDNA were ligated with the TT adapter. Fifteen μl each of the adapterized HL60 cDNA and HL60/TLK286 cDNA were mixed with 30 μl of 2x HB buffer in a final volume of 60 μl, denatured at 95°C for 5 min and annealed at 68°C for 20 hours. The reassociated DNA was used as a template for the PCR direct labeling. To generate the probe of HL60 DNA, a PCR reaction was set up with 0.5 μl of the reassociated DNA, 5 μl of 10x Clontech PCR buffer, 1 μl of dNTPS (10 mM dATP, dTTP, and dGTP each, 6 mM dCTP), 4 μl of FluoroLink Cy3-dCTP (Amersham Pharmacia), 4.5 μl of 10 μM CT primer, 1 μl of Clontech cDNA polymerase and 34 μl of ddH$_2$O. In the PCR reaction for the probe of HL60/TLK286 DNA, Cy5-dCTP and TT primer were used instead of Cy3-dCTP and CT primer. The reaction cycling conditions were 72°C for 5 min (for filling in the adapter ends), 94°C for 1 min, then 30 cycles of 94°C for 30 s, 62°C for 30 s, 72°C for

60 s, then 72°C for a final extension. Three PCR reactions were set up for each probe. The Cy3 (HL60) and Cy5 (HL60/TLK286) PCR products were purified with Qiagen PCR purification kit, reduced to a final volume of 7.5 μl with a speed vac, mixed with 3.75 μl of 20x SSC, 0.75 μl of 10% SDS, 1.5 μl of 1 μg/μl salmon DNA and 1.5 μl of 50x Denhardt's solution, denatured at 95°C for 5 min, cooled on ice and incubated at 42°C for 15 min. The denatured Cy3 (HL60) and Cy5 (HL60/TLK286) DNA were mixed and loaded onto a microarray chip. The hybridization and washing conditions were the same as for regular microarray. Three replicates were performed on the first and second sets of human microarray chips containing 10,368 genes each.

RT-PCR

Quantitative RT-PCR was used to verify the results of ADGE microarray. Primers for RT-PCR were designed with Software OLIGO 4.0 based upon the sequences of the genes corresponding to the identified spots on the chip. The total RNA samples of HL60 and HL60/TLK286 were reverse-transcribed with Superscript II reverse transcriptase. The cDNA templates of the two samples were normalized with beta-actin. Three levels of dilution were made for templates. For each gene, specific PCR cycle conditions were selected to optimize the levels of differential expression. The bands of PCR products were quantified with NIH Image 1.62 and ratios were calculated.

Analysis of microarray data

The spots from the microarray images were quantified with ImaGene4.1 (Biodiscovery, Los Angeles, CA). The Cy3 and Cy5 data were integrated into a data set and transformed with GeneSight3.0 (Biodiscovery) by using local background correction and logarithm of base 2 for the verification experiment of ratio magnification. The following sequence was used for regular and ADGE microarray: local background correction, removal of flagged spots, logarithm of base 2, ratio calculation and linear regression normalization. The transformed data were exported into Microsoft Excel. The three replicates were combined and MA plots were constructed [21,22]. M = $\log_2(Cy5/Cy3)$ = $\log_2 Cy5 - \log_2 Cy3$; A = $\log_2((Cy5 * Cy3)^{1/2})$ = $(\log_2 Cy5 + \log_2 Cy3)/2$. A represents the intensity of a spot. M represents the ratios in the power of 2, with positive values for up-regulated genes (Cy5/Cy3), negative values for down-regulated genes (Cy3/Cy5), zero for unchanged genes. In addition, variances of Cy3 and Cy5 and the p-values of t test were calculated for each gene. Confidence levels were calculated from the p-values. Genes with significantly altered expression were selected based on a threshold of ratios and confidence level.

Authors' contributions

ZJC participated in the design of the study, carried out major experiments, performed the data analysis and drafted the manuscript. LG participated in the design of the study and carried out part of the experiments. WD participated in the design of the study. KEI carried out part of the experiments. KDT participated in the design of the study and coordination. All authors read and approved the final manuscript.

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