ORIGINAL ARTICLE

Expressive genomic hybridisation: gene expression profiling at the cytogenetic level

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Aims: To describe a cytogenetic technique suitable for the rapid assessment of global gene expression that is based on comparative genomic hybridisation (CGH), and to use it to understand the relation between genetic amplifications and gene expression.

Methods: Whereas traditional CGH uses DNA as test and reference in hybridisations, expressive genomic hybridisation (EGH) uses globally amplified mRNA as test and normal DNA as reference. EGH is a rapid and powerful tool for localising and studying global gene expression profiles and correlating them with loci of genetic amplifications using traditional CGH.

Results: EGH was used to correlate genetic amplifications detected by CGH with the expression profile of two independent cell lines—Colo320 and T47D. Although many amplifications resulted in overexpression, other amplifications were partially or completely silenced at the cytogenetic level.

Conclusion: This technique will assist in the analysis of overexpressed genes within amplicons and could resolve a controversial issue in cancer cytogenetics; namely, the relation between genetic amplifications and overexpression.

Comparative genomic hybridisation (CGH) is a well established, wide scanning technique used to study and localise genetic aberrations such as deletions, amplifications, and chromosomal copy number changes in cancer.^{1,2} CGH has significantly enhanced our knowledge on the extent and the role of chromosomal aberrations in cancer and cancer progression. To date, there is a large body of published CGH studies on cancer, reporting widespread genetic aberrations and, in at least some, associating these aberrations with prognosis.^{3–5} Amplifications detected by CGH are of particular interest in cancer research because they are traditionally thought of as housing important oncogenes that are overexpressed in cancer.^{6,7} Indeed, several studies have localised and isolated oncogenes based on data generated by CGH.⁸ However, with the development of more refined techniques, such as microarrays, the issue of whether whole amplicons are overexpressed or just house a few genes that are overexpressed has become controversial. For example, using cDNA microarrays, Forozan and colleagues⁹ found several overexpressed genes that were localised within amplicons identified by CGH, whereas Platzer and colleagues¹⁰ found a limited number of genes with low levels of overexpression and a larger number of underexpressed genes within amplicons detected by CGH. These discrepancies are understandable because the CGH and cDNA microarray techniques have different sensitivities. Whereas CGH identifies large areas of chromosomal aberration, which house many genes that have not yet been identified, microarrays identify single gene copy number changes or alterations of expression in single genes. The selection of genes for study in microarray experiments is governed by the manufacturer, space, and the availability of sequence data. Therefore, there is currently a need for a method that correlates genomic expression with expression profiles at the cytogenetic level. Here, we describe such a method, which we have termed expressive genomic hybridisation (EGH), and which spans the gap between traditional CGH and microarrays. Whereas traditional CGH uses DNA strands as test and reference in hybridisations, EGH makes use of globally amplified mRNA as test and normal DNA as reference. This gives EGH a twofold advantage: (1) it identifies globally over-represented cDNA in the test sample, based on chromo-

somal localisation; and (2) it can be used to correlate the expression profile with amplification information generated by traditional CGH. Therefore, specific genes found to be over-represented by EGH can be analysed further by cDNA microarrays, and the choice of genes for spotting can be guided by the EGH results. Expression profiling at the cytogenetic level has been previously reported using degenerate oligonucleotide primer-polymerase chain reaction (DOP-PCR) amplified cDNA.¹¹ However, our approach differs in three major ways. First, our cDNA synthesis uses the Clontech switching mechanism at the 5' end of the RNA transcript (SMART[®]) in the first strand synthesis to generate high yield full length cDNA from mRNA only and not DNA, so that DNase treatment of RNA is not required.¹² Second, the reference used is normal DNA and not cDNA. Because the cDNA from normal tissue can have a different expression profile to the test DNA, its use as a reference for quantification can be a limiting step. Third, whereas DOP-PCR amplifies rare sequences, the method reported here maintains the relative levels of expression of the different transcripts.¹³ This is important because only signals that are highly overexpressed are visualised using our method.

"There is currently a need for a method that correlates genomic expression with expression profiles at the cytogenetic level"

We have used CGH and EGH, respectively, to study and correlate genetic amplifications with expression in T47D and Colo320 cell lines and show that, although some genetic amplifications identified by traditional CGH are over-represented in the total cDNA population, in many chromosomes these amplifications are partially or completely silenced at the cytogenetic level.

Abbreviations: CGH, comparative genomic hybridisation; DOP, degenerate oligonucleotide primer; EGH, expressive genomic hybridisation; PCR, polymerase chain reaction; SSC, saline sodium citrate

Figure 1 Chromosomal imbalances detected by comparative genomic hybridisation in the Colo320 cell line. The profiles represent average green to red fluorescence intensity along chromosomes collected from at least seven metaphase spreads with corresponding chromosome ideograms. Mean ratios are shown as thick lines, and the 99% confidence intervals of the mean are shown in green for values significantly above the mean and red for values below the mean. Green bars denote increased chromosome copy numbers or amplifications, whereas red bars represent reduced chromosome copy numbers or deletions. Numbers in brackets indicate chromosome counts.

MATERIALS AND METHODS

DNA extraction

DNA was extracted from the two cell lines Colo320 and T47D using the Purgene kit (Gentra Systems, Minnesota, USA), according to the manufacturer's protocol. In brief, the cell pellet was lysed with 600 μ l of Purgene cell lysis solution with the addition of 20 μ l proteinase K (20 mg/ml; Gibco BRL, Gaithersburg, Maryland, USA). The samples were digested at 56°C for 16 hours. To obtain RNA free DNA, samples were treated with 3 μ l RNase A (4 mg/ml; Gentra Systems), according to the manufacturer's instruction. Proteins were precipitated by the addition of 200 μ l protein precipitation solution. Samples were then vortexed and centrifuged at 16 000 $\times g$ for five minutes. The supernatant was transferred to a 1.5 ml eppendorf tube containing 600 μ l isopropanol. The tube containing the mixture was inverted and the samples were centrifuged at 16 000 $\times g$ for five minutes. The DNA pellets obtained were washed with 70% alcohol and vacuum dried. The DNA pellet was dissolved in 70 μ l TE buffer (10mM Tris HCl, pH 7.0, 1mM EDTA, pH 8.0). The DNA concentration was measured with an ultraviolet spectroscope. Normal DNA for CGH experiments was extracted from the blood of a healthy donor using the above protocol, except that the red blood cells were lysed with Purgene RBC lysis solution (Gentra Systems). The white blood cells were lysed with Purgene cell lysis solution (Gentra Systems).

Labelling of DNA for CGH

Cell line DNA (2 μ g aliquots) was labelled using biotin nick translation reagent (Roche, Hamburg, Germany), according to manufacturer's instructions. The labelling reaction was carried out at 15°C for 75 minutes and the reaction was stopped by the addition of 2 μ l of 0.5M EDTA (pH 8.0). Sam-

ples were then heated to 72°C for 10 minutes. Normal DNA was labelled as above with digoxigenin nick translation reagent (Roche, Mannheim, Germany).

Comparative genomic hybridisation

The test and reference labelled DNAs were mixed in equal concentrations (~2.0 μ g) and precipitated with 100 μ g human Cot1 DNA (Invitrogen). The CGH probe was pelleted by centrifugation, washed with 70% alcohol, and vacuum dried. The probe was dissolved in 16 μ l CGH hybridisation buffer (50% dextran sulfate in 2 $\times$ saline sodium citrate (SSC) containing 50% formamide). The probe was denatured at 75°C for 10 minutes and reannealed at 37°C for one hour. CGH metaphase slides were obtained from Vysis Inc (Illinois, USA) and metaphases were marked under a phase contrast microscope and incubated in denaturation solution (70% deionised formamide in 2 $\times$ SSC) at 73°C for three minutes. The slides were then dehydrated in a series of alcohol (70%, 80%, and 100%) for three minutes each and left to dry at 37°C for 10 minutes. After applying the probe, slides were incubated at 37°C in a humid environment for 72 hours. The slides were finally washed with 70% formamide in 2 $\times$ SSC buffer and stained with fluorescent antibodies and diaminodiphenylindole (DAPI), as described previously.² Images were captured by CCD camera (JVL, JAI Corporation, Japan) using DAPI, fluorescein isothiocyanate, and Texas red filters mounted on Axioplan 2 imaging system (Carl Zeiss, Hamburg, Germany). The images were then analysed by ISIS® software (Metasystems, Hamburg, Germany). Typically, 10 to 20 metaphases were captured using the appropriate filters. The images were digitally stored and the green to red and red to green ratios were calculated. Amplifications were scored if the median

Figure 2 Chromosomal imbalances detected by comparative genomic hybridisation in the T47D cell line. The profiles represent average green to red fluorescence intensity along chromosomes collected from at least seven metaphase spreads with corresponding chromosome ideograms. Mean ratios are shown as thick lines, and the 99% confidence intervals of the mean are shown in green for values significantly above the mean and red for values below the mean. Green bars denote increased chromosome copy numbers or amplifications, whereas red bars represent reduced chromosome copy numbers or deletions. Numbers in brackets indicate chromosome counts.

green to red ratio was above or equal to the 1.25 threshold, and deletions (only for CGH) were below or equal to the 0.75 threshold. In all experiments, separate normal versus normal DNA hybridisation controls were also included. A genetic aberration was scored if the probability representing the 99% confidence interval of the tumour versus the normal profiles was out with the 99% confidence interval of the normal versus normal control profiles.

RNA extraction

Cell lines at 70% confluency were harvested and then transferred to DEPC treated 1.5 ml eppendorf tubes. The cells were washed twice with phosphate buffered saline and centrifuged at $165 \times g$ for five minutes at 4°C . The cells were lysed with 500 μl of Trizol (Life Technologies, Hamburg, Germany). Chloroform (500 μl) was then added, and the mixture was vortexed and then centrifuged at 4°C , $6800 \times g$ for 30 minutes. The supernatant was transferred into a clean DEPC treated 1.5 ml eppendorf tube and an equal volume of isopropanol was added for precipitation. The mixture was kept on ice for 15 minutes and then centrifuged at 4°C , $20\,800 \times g$ rpm for 30 minutes. The RNA pellet was washed with 70% alcohol, vacuum dried, and dissolved in 70 μl of DEPC treated water.

Full length cDNA synthesis and amplification

Total RNA (1.0 μg) was reverse transcribed into cDNA using the primers 5'-AAGCAGTGGTATCAACGCAGAGTACGCGGG-3' and 5'-AAGCAGTGGTATCAACGCAGAGTACT₍₃₀₎N₍₁₎-3' (Clontech, Palo Alto, California, USA). The samples were first denatured with the above two primers at 70°C for two minutes and mixed with 5 $\times$ first strand buffer (250mM Tris/HCl, 365mM KCl, 30mM MgCl_2), dithiothreitol (20mM), 50 $\times$ dNTP, and PowerScript reverse transcriptase (Clontech). The

reaction mixture was incubated at 42°C for one hour. The first strand reaction was diluted with 40 μl of TE buffer (10mM Tris, 1mM EDTA). The reaction was stopped by heating at 72°C for seven minutes. cDNA amplification and labelling were performed in the next step by Advantage 2 PCR (Clontech). A 2 μl aliquot of the cDNA sample was added to the master mix containing 10 $\times$ Advantage PCR buffer, 50 $\times$ dNTP (10mM), 0.1mM biotin 16-dUTP (Roche Boehringer), 50 $\times$ Advantage 2 polymerase mix, and a 10 μM of 5' PCR primer (5'-AAGCAGTGGTATCAACGCAGAGT-3'; Clontech) in a 100 μl total volume. PCR was performed using initial denaturation at 95°C for one minute, followed by 20 cycles of 95°C for five seconds, 65°C for five seconds, and 68°C for six minutes. A 10 μl aliquot of the PCR product was run on a 1.2% agarose gel, and produced a smear of cDNA ranging from 0.4 to 6 kbp.

Expressive genomic hybridisation

Biotin labelled amplified cDNA from the cell line and the digoxigenin labelled normal reference male DNA were mixed in equal parts and precipitated with 100 μg of human Cot-1 DNA (Invitrogen). The probe was dissolved in CGH hybridisation buffer (50% formamide, 50% dextran sulfate in $2\times$ SSC), denatured, reannealed, and hybridised to denatured normal metaphase CGH slides using the procedure described above. To validate this method, we included two control experiments. In the first control, the labels were reversed: the cell line cDNA was labelled with digoxigenin and the normal DNA was labelled with biotin, so that the same EGH profiles would be expected, but in reverse colours. In the second control, we hybridised the cell line cDNA labelled with biotin with the same cell line cDNA labelled with digoxigenin to ensure maximum specificity of the signals. The slides were processed as described for CGH. The images were digitally stored and the green to red and red to

green ratios were calculated. Overexpression was scored if the median green to red ratio was above or equal to the 1.25 threshold and underexpression when the green to red ratio was below or equal to the 0.75 threshold.

RESULTS AND DISCUSSION

CGH detected several genetic aberrations in the Colo320 cell line, including amplification of 1p21–q24, 2p25.3–q22, 3q13.1–q29, 4p16–q26, 6p23–p25, 6q15–q27, 8q23–q24.3, 9q21.3–q34.3, 11p12–15.5, 12p13.3–q15, 12q23–24.2, 13p13–q34, 14p11.2–13, 17q21–25, 18q11.1, and 22p12–q12.3, in addition to an increase in copy number of chromosomes 16 and 20. Deletions detected by CGH in this cell line were at 5p14–15.3, 5p11, 5q33.3–ter, 7p11.1–p13, 8q12, 10p11.2–12.3, 10p13–14, 11q12–13.1, 11q23.1–23.3, 11q24, 17p11.2–12, and 21p13–ter (fig 1). The profile generated is in accordance with previously published data.^{14–16} Similarly, the CGH profile of the T47D cell line showed amplification of 1q11–q43, 3p21.3–25, 3q24–29, 5p15.3–q11.3, 5q33.1–35.3, 6p22.1–p25, 7p21.1–q21.1, 8p12–qter, 10q11.2–24.2, 11p15–qter, 12q11.1–qter, 14p13–q23, 15q21.3–qter, 17q11–q21.3, and 21q22.2–qter, in addition to increased copy number of chromosome 20. The areas containing deletions in this cell line were 2p21–pter, 3p11.1–14.1, 3q13.1–13.3, 9p21–p23, 12p12.3–pter, 16p11.2–13.1, 18q12.3–qter, and 19q13.1–13.2 (fig 2). Although CGH analysis on this cell line has not been reported, we chose to use it because its DNA and expression profiles have been previously established using microarrays.^{15–17} We then generated cDNA probes from the same cell lines (as described earlier) and applied them to metaphase chromosomes. SMART cDNA synthesis technology has not been used previously to generate global hybridisation probes. DOP-PCR has been used extensively for the amplification of nucleic acids in CGH,^{18–19} but because contaminating genomic DNA can be amplified by DOP-PCR, cDNA amplified by DOP-PCR should be treated with DNase before amplification. In contrast, the SMART method requires no prior DNase treatment because one of the primers has a poly T chain that anneals to mRNA only. In addition, SMART cDNA amplification maintains the relative degrees of expression of the different transcripts.¹³

To test the size range of the mRNA amplification procedure, the PCR products were run on a 1.2% agarose gel and showed smeared fragments ranging from 400 to 6000 bp (fig 3). To ensure that DNA is not amplified by this method, an RNA free DNA sample was also amplified and no product was seen, as was expected (fig 3, lane 3). Because the hybridisation of regular cDNA on to metaphase slides produced no detectable signals, we first tested whether the amplified cDNA can generate detectable signals on corresponding chromosomes. To do this, the amplified test samples were hybridised to metaphase slides without reference nucleic acid. Figure 4A shows the highly efficient hybridisation signals seen using this method. To quantitate the expression relative to a reference point, normal genomic reference DNA was co-hybridised with the amplified cDNA (fig 4B). CGH profiles generated from the Colo320 and T47D cell lines were then compared with the results obtained by EGH from the same cell lines (fig 4C). The results of copy number changes/amplifications versus expression profiles for the Colo320 and T47D cell lines are summarised in figs 5 and 6, respectively. The profiles generated can be subclassified into the following categories of chromosome regions: (1) amplified and totally or partially expressed; (2) not amplified but highly expressed; and (3) amplified but not expressed. For the Colo320 cell line, category 1 areas comprised: 1p21–1p13.3, 1q12–1q24, 2p13–p22, 2q12, 3q21–29, 4p14–q26, 6q15–q18, 6q22, 6q23.3–q25, 8q23–24.3, 12p13–p11.2, 13p11.2–q33, 16q11.2–q12.2, and 17q21–q25; category 2 areas comprised: 2q24.1–q33, 5p13–p12, 5q13–q23.3, 7q22–31.3, 12q21.3–q23, 14q11.2–q24, and 17q11.2–q21; and category 3 areas comprised: 1p13.3–q12, 2p25–p22, 2p13–q12, 2q12.3–q22, 3q13.1–q21, 4p16–p14, 6p25–23, 6q17–

Figure 3 Ethidium bromide stained agarose gel of amplified mRNA obtained from the T47D cell line. Lane 1, digoxigenin labelled cDNA. Lane 2, biotin labelled cDNA. Lane 3, RNase treated DNA amplified by SMART technology. Lanes 4, 5, and 6 are unlabelled cDNA. Lane 7, 100 bp size marker.

q22, 6q22.3–23.3, 6q25–q27, 8q24.1–q24.3, 9q21.3–q34.3, 11p15.5–p12, 12p13.3–p13, 12p11.2–q14, 12q23–q24.2, 13p13–p11.2, 13q33–q34, 14p13–q11, 16p13.3–q11.2, 16q13–q24, 20p13–q11.2, and 22p12–q12 (fig 5). Similarly, for the T47D cell line category 1 chromosomal areas comprised: 1q22–q42, 3p24–p22, 3q25–q29, 5p15.3–q11.2, 6p22, 6q24–q25, 7p15–p12, 8p12–p11.2, 8q13–q24.1, 10q21.2, 11p15.1–p11.2, 11q14–q23, 11q24–q25, 12q13–q24.1, 14p12–q23, 15q21–q24, and 15q26; category 2 areas comprised: 5q11.2–q13, 7q21–q22, 12p12–p11.2, 13q14–q21.1, and 21q11–q21.3; and category 3 areas comprised: 1q12–q22, 1q42.1–q43, 3p25–24.1, 3p22–p21.3, 3q24–q25.1, 5q33–q35, 6p25–p22.1, 7p21.1–p15, 7p12–q21.1, 8q11.2–q13, 8q24.1–24.3, 10q11.2–q21, 10q21.3–24.1, 11p15.2–p15.1, 11p11.2–q14, 11q23.3, 11q24–q25, 12q12–q13, 12q24.1–q24.3, 15q24–q25.5, 15q26–qter, 17q11.2–q21.3, 20q11.2–qter, and 21q22–qter (fig 6).

For both cell lines, the expression profiles were confirmed by reverse labelling of the cDNA and the reference DNA (fig 4B,C). In addition, neither of the cell lines expressed sequences that localise to the Y chromosome because they were developed from female patients (fig 4B, see Y chromosome profile). As expected, none of the deleted areas detected by CGH in both cell line was overexpressed.

“Expressive genomic hybridisation can be used to identify and localise important genes that could have been missed as potential oncogenes by comparative genomic hybridisation”

Our results demonstrate that genetic amplifications closely correlate with gene overexpression, which is in accordance with previously published data. However, the results also show that, in many chromosomes, amplifications do not necessarily

Figure 4 Comparative genomic expressive hybridisation detection and quantification. (A) Amplified cDNA hybridised to chromosomes without reference DNA. The left hand image shows hybridisation of cDNA obtained from the Colo320 cell line to chromosome 8q24 (arrow), which contains the locus for the c-myc oncogene, and denotes overexpression of that locus. The right hand image represents hybridisation of cDNA obtained from the T47D cell line to chromosome 3. (B) An example of expressive genomic hybridisation (EGH) using amplified cDNA from the T47D cell line labelled with digoxigenin (red) co-hybridized with normal male reference DNA labelled with biotin (green). Red bars indicate overexpression along various chromosomes, whereas green bars indicate underexpression. The profiles below show the red to green intensities along chromosomes obtained from at least 10 metaphases. (C) Comparison between EGH (right) and comparative genomic hybridisation (left) profiles and chromosome paints from chromosomes 2, 3, and 8 of the Colo320 cell line and chromosome 14 of the T47D cell line, in that order from top to bottom. Notice the overexpressed sequences hybridising to the chromosome 2q24-q33 region (right), which is not amplified (left).

Figure 5 Chromosome ideograms; summary of amplified and expressed sequences obtained by comparative genomic hybridisation and expressive genomic hybridisation from the Colo320 cell line. Yellow areas represent amplified and expressed loci. Green areas represent amplified but not expressed loci and red areas represent expressed loci without evidence of amplification. Uncoloured areas are not involved in expression or amplification.

result in overexpression. It could be that these areas contain a few genes that are overexpressed but their detection is beyond the limits of this method. Nevertheless, the fact that overexpression was clearly demonstrated in many amplified chromosomal regions but not in others suggests a cellular mechanism of gene expression control. This result is supported by previous work, which found a limited number of genes with low levels of overexpression or even larger number of genes overexpressed within amplicons detected by CGH.^{10 16 20} These controversies will remain until the development of single arrays that carry the complete human genome. Until then, microarray based

strategies suffer from an important limitation; namely, that they can only provide information on genes that have been previously selected for spotting on arrays. Although other published techniques have been described to circumvent such limitations,²¹ the technique described here offers a method for focusing on and selecting genes based on their chromosomal location to be investigated further by microarrays. In addition, because EGH localises overexpressed transcripts to particular chromosomes without them being identified as amplicons, this method can be used to identify and localise important genes that could have been missed as potential oncogenes by CGH.

Figure 6 Chromosome ideograms; summary of amplified and expressed sequences obtained by comparative genomic hybridisation and expressive genomic hybridisation from the T47D cell line. Yellow areas represent amplified and expressed loci. Green areas represent amplified but not expressed loci and red areas represent expressed loci without significant evidence of amplification. Uncoloured areas are not involved in expression or amplification.

EGH allows the development of a global expression signature for a cell line or tissue at the cytogenetic level. Because ultimately expression is what determines the characteristics of individual tumours, we envisage that this technique could be used for the localisation of expression sequences and their relevance to tumour progression. This is important in view of our finding that some amplified sequences are not necessarily overexpressed and that some overexpressed sequences are generated by mechanisms other than genomic amplification.

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Take home messages

- We used expressive genomic hybridisation (EGH) to correlate genetic amplifications detected by comparative genomic hybridisation with the expression profile of two independent cell lines
- Many amplifications did result in overexpression, but other amplifications were partially or completely silenced at the cytogenetic level
- EGH can provide a global expression signature for a cell line or tissue at the cytogenetic level and could be useful for the localisation of expression sequences and determining their relevance to tumour progression

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