Detection of Gene Amplification by Genomic Hybridization to cDNA Microarrays

Mervi A. Heiskanen, Michael L. Bittner, Yidong Chen, Javed Khan, Karl E. Adler, Jeffrey M. Trent, and Paul S. Meltzer

Cancer Genetics Branch, National Human Genome Research Institute, NIH, Bethesda, Maryland 20892 [M. A. H., M. L. B., Y. C., J. K., J. M. T., P. S. M.], and DuPont NEN Life Science Products, Inc., Boston, Massachusetts 02118 [K. E. A.]

Abstract

Gene amplification is one of the major mechanisms of oncogene activation in tumorigenesis. To facilitate the identification of genes mapping to amplified regions, we have used a technique based on the hybridization of total genomic DNA to cDNA microarrays. To aid detection of the weak signals generated in this complex hybridization, we have used a tyramide-based technique that allows amplification of a fluorescent signal up to 1000-fold. Dilution experiment suggests that amplifications of 5-fold and higher can be detected by this approach. The technique was validated using cancer cell lines with several known gene amplifications, such as those affecting MYC, MYCN, ERBB2, and CDK4. In addition to the detection of the known amplifications, we identified a novel amplified gene, ZNF133, in the neuroblastoma cell line NGP. Hybridization of NGP cDNA on an identical array also revealed over expression of ZNF133. Parallel analysis of genomic DNA for copy number and cDNA for expression now provides rapid approach to the identification of amplified genes and chromosomal regions in tumor cells.

Introduction

Chromosomal anomalies resulting in gain or loss of genetic material are frequent in tumor cells. These changes may result in alterations in the level of expression of numerous genes. Indeed, gene amplification is one of the most common mechanisms for oncogene activation in solid tumors. It is therefore of considerable interest to develop strategies for identifying amplified genes and determining their expression levels in cancer. Until recently, gene amplification has been detected by DNA-based techniques (PCR or Southern blot) or by molecular cytogenetic techniques (FISH with gene-specific probes). These techniques are inherently restricted to the analysis of previously known amplified genes. In contrast, genome-wide scanning of amplified chromosomal regions with CGH (1) has become an important technique for the detection of amplified regions in tumor DNA. However, CGH has limited sensitivity and resolution (2). In addition, the identification of the specific target gene within an amplicon defined by CGH remains daunting because of the limited mapping resolution provided by the metaphase chromosomes.

DNA microarray technology offers the possibility to replace the target metaphase chromosomes with arrays of DNA clones on a microscope slide. Arrayed fragments of cloned genomic DNAs have been used for this purpose (3, 4). These CGH microarray techniques allow amplification detection on the resolution level equal to the length of the arrayed DNA clones (~100 kb). Genomic microarrays have been applied to amplicon mapping (4), but the technique can also be used for rapid surveys of known copy number alterations in tumor samples. In principle, a further increase in resolution can be obtained by using arrayed cDNAs rather than genomic DNA. This approach is particularly attractive because of the availability of thousands of accurately mapped cDNAs. Furthermore, expression analysis can be carried out in parallel on the same microarray slides, enabling a correlation of copy number and gene expression. However, signal intensities in genomic hybridizations are proportional to the length of the target DNA (4). Reproducibly achieving a measurable hybridization signal from total genomic DNA hybridized to targets covering only 0.5–2 kb is difficult and requires a signal detection system with high sensitivity and low background. An approach for CGH on cDNA microarrays was reported recently by Pollack et al. (5) using directly labeled fluorochrome probes. We report here an alternative technique that also uses cDNA arrays prepared for expression studies. Our results indicate that this technique is reproducibly capable of detecting gene amplifications of 5-fold or higher. Finally, the suitability of the technique for genome-wide screening of amplified and overexpressed genes was tested by hybridizing both genomic neuroblastoma DNA and mRNA on a microarray containing 1400 genes. In addition to known amplifications on 2p and 12q, a previously unrecognized amplicon on 20p containing a zinc finger gene (ZNF133) was identified.

Materials and Methods

Preparation of cDNA Microarrays

cDNA microarrays were prepared as described previously (6). To validate the technique, microarrays containing 14 different cDNAs representing genes known to be amplified in cancer cell lines were printed (CDK4, MDM2, OS4, OS9, MYCN, MYC, MYCL, EGFR, AKT2, ERBB2, AIB1, IGF1R, CLND, and SAD) for screening of unknown amplified genes, an array containing 1400 cDNAs was used (6).

Genomic Hybridization, Signal Detection, and Amplification

Total genomic DNA was labeled with biotin by nick translation for 2.5 h at 15°C. The fragment size of the labeled probe was between 400-2000 bp. Unincorporated nucleotides were removed using Micro Bio-Spin 6 Chromatography Columns (Bio-Rad, Hercules, CA). Hybridization mixture (10 μl) was composed of 1 μg of biotinylated probe, 40 μg of Cot-1 DNA (Life Technologies, Inc., Rockville, MD) and 8 μg of poly dA in 3X SSC (0.15 M NaCl, 0.015 M sodium citrate)/0.01% SDS. After denaturation, the hybridization mixture was added on the slide and hybridized in a hybridization chamber at 65°C over night. Slides were washed in 0.5X SSC/0.01% SDS, 0.06X SSC/0.01% SDS, and 0.06X SSC at room temperature for 5 min each. Hybridization signals were developed using tyramide reagents (Renaissance TSA-indirect ISH; DuPont NEN Life Science Products). Slides were blocked using 10% goat serum in TN blocking buffer [0.1 M Tris (pH 7.6), 0.15 M NaCl]. Hybridization was detected by first incubating the slide with streptavidin conjugated with horseradish peroxidase (1:100 in TN-10% goat serum), followed by signal amplification with biotinyl tyramide (1:50 in reaction buffer with 1% blocking reagent). Biotinyl tyramide was detected by streptavidin conjugated with Cy3 (1:500 in TN-10% goat serum). Between and after incubations, slides were washed with TNT buffer [0.1 M Tris-HCl (pH 7.5),

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1 To whom requests for reprints should be addressed, Cancer Genetics Branch, National Human Genome Research Institute, NIH, 49 Convent Drive, MSC 4470, Bethesda, MD 20892.

2 The abbreviations used are: FISH, fluorescence in situ hybridization; CGH, comparative genomic hybridization.
0.15 M NaCl, and 0.05% Tween 20 for 3 × 1 min. All of the incubations and washings were done at room temperature.

cDNA Hybridization for Gene Expression Analysis

Hybridization of NGP cDNA on a microarray containing 1400 genes has been described in detail previously (6).

Image Analysis and Outlier Detection

Amplification Intensity. A gray scale fluorescent image for each microarray slide was obtained from a confocal scanning microscope. DNA target segmentation and signal detection methods were then used to determine the actual target regions, average signal intensities, and local background intensities (7). The background subtracted average signal intensity was reported as the hybridization intensity.

Fold Increase. An iterative amplification intensity outlier detection algorithm was then applied as follows:

(a) Assuming there were N cDNA targets presented in microarray slides, we first sorted all intensities \( I_k \) in ascending order, \( I_1 \geq I_2 \geq \ldots \geq I_N \). We then partitioned intensities into two groups \( (I_1, \ldots , I_{N-m}) \) and \( (I_{N-m+1}, \ldots , I_N) \). Initially, we chose \( m = N/2 \).

(b) The discordance test (for a single outlier in a normal sample with \( m \) and \( s \) unknown) was performed for the first amplification intensity from the second group:

\[
\frac{\mu_1 - I_{N-m+1}}{\sqrt{\left(\sigma_1^2 + \sigma_s^2\right)}}
\]

where

\[
\mu_1 = \frac{\sum_{i=1}^{N-m} I_i}{N - m}
\]

and

\[
\sigma_1 = \frac{\sum_{i=1}^{N-m} (I_i - \mu_1)^2}{N - m - 1}
\]

and \( \sigma_s \) is the SD of local background at the same location of \( I_{N-m+1} \). The test statistic \( T \) can be converted to Student’s \( t \) test with \( N - 2 \) degrees of freedom [Barnett and Lewis (8)]. As an example, the critical value for \( n = 40 \) and \( \alpha = 1\% \) discordance, \( t \) must be >3.24.

3) If the \( I_{N-m+1} \) was not an outlier, let \( m \leftarrow m + 1 \), and then repeat step 2 until the first outlier intensity was obtained.

If at least one outlier was obtained, the amplification intensities were partitioned into two groups: (a) negative targets that exhibit no significant amplification intensities; and (b) positive targets of which intensities were statistically different from those from the negative group. The fold increase \( r_k \) was then calculated for all genes in the positive target group \( (I_{N-m+1}, \ldots , I_N) \) by \( r_k = I_k/\mu_k \) for \( k = N - m + 1, \ldots , N \).

P Value. To further assess the significance of each reported positive amplification intensity, \( P \) from the aforementioned discordance \( t \) test statistic (8) can be calculated.

In Situ Hybridization

A BAC clone specific for ZNF133 (169o05) was screened from a human BAC library (Research Genetics, Inc. Huntsville, AL) using primers specific for marker W1–18789.3 The probe was labeled with Spectrum Orange by random priming (BioPrime DNA Labeling System; Life Technologies, Inc.). FISH-based copy number determination for the ZNF133 region included a fluorescein-labeled satellite probe for chromosome 20 (Oncor).

\(^3\) Internet address: http://www.genome.wi.mit.edu.

\(^4\) A. Kallioniemi, personal communication.
with the NGP dilution experiment (Fig. 1), which suggested that the sensitivity of detection is ~5-fold.

To determine whether previously unknown amplification could be identified this technique, we hybridized NGP genomic DNA to a 1400 element cDNA microarray. In addition to the known amplified genes, a novel gene amplification was identified in this experiment (Fig. 3). The signal intensity of an expressed sequence tag representing ZNF133 was observed to be significantly increased (8-fold over the mean). ZNF133 has been mapped previously to 20p11.2 by in situ hybridization (13). CGH of NGP on metaphase chromosomes also indicated the amplification of this chromosomal region (data not shown). To verify the amplification, a ZNF133 BAC clone was hybridized on NGP cells in situ. The signal copy number in interphase nuclei was heterogeneous, ranging from 2 copies up to 22 copies of ZNF133 per cell. The average copy number calculated from 50 cells was 6.6. In contrast, two copies of the chromosome 20 centromere reference probe were detected in all nuclei.

In two-color hybridization of NGP cDNA (red) relative to normal fibroblast cDNA (green), the red:green ratio of the ZNF133 was 3.4, indicating a significant increase in expression of this amplified gene relative to the reference probe (6). This demonstrates the ability to perform both expression and copy number analysis on the same set of microarrays.

**Discussion**

Compared with the previously published CGH microarray techniques where genomic DNA is hybridized on arrayed genomic clones (3, 4), hybridization on cDNA arrays offers significant advantages. The most important is the possibility to directly identify amplified genes rather than amplified genomic regions. Another advantage is the ability to do expression studies on the same arrays using the standard cDNA microarray approach. Finally, thousands of mapped cDNAs are readily available, which facilitates amplicon mapping and identification of new cancer genes.

The complexity of the probe and the small sizes of the arrayed target cDNAs (0.5–2 kb) place high demands on the sensitivity of the system. Using tyramide-based signal amplification (14), it is possible to enhance fluorescent signals up to 1000-fold. Deposition of biotin tyramides has been applied previously for the amplification of in situ hybridization signals (15). We show here that the peroxidase-mediated deposition of biotin tyramide can also be applied on high sensitivity detection of gene amplification on cDNA microarrays to detect gene amplifications of 5-fold or greater. Although we have tested the addition of a second tyramide reagent to provide two-color CGH, there was excessive cross-talk between tyramide reagents under the conditions necessary for genomic hybridization. Nonetheless, the tyramide method described here consistently generates significant signal intensities necessary for a screening technique for gene amplification.

In the hybridization of NGP DNA to a 1400-element cDNA microarray, we identified the known 2p and 12q amplicons as well as a novel 20p amplicon containing a zinc finger gene ZNF133. This gene belongs to the family of Kruppel-related zinc finger genes that have been connected with transcriptional repression (13). Amplification

**Table 1 Gene amplifications detected by genomic hybridization of cancer cell line DNA on cDNA microarrays**

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Nonpositive mean intensity</th>
<th>Gene</th>
<th>Amplified gene intensity</th>
<th>Fold change</th>
<th>( p^a )</th>
</tr>
</thead>
<tbody>
<tr>
<td>NGP</td>
<td>6861.00</td>
<td>MYCN</td>
<td>42138.00</td>
<td>6.14</td>
<td>0.00</td>
</tr>
<tr>
<td>SAS</td>
<td>23302.00</td>
<td>CDK4</td>
<td>19192.00</td>
<td>2.79</td>
<td>0.05</td>
</tr>
<tr>
<td>MDM2</td>
<td>20509.00</td>
<td>OS4</td>
<td>36832.00</td>
<td>5.37</td>
<td>0.02</td>
</tr>
<tr>
<td>HL60</td>
<td>1097.20</td>
<td>MYC</td>
<td>30675.00</td>
<td>28.20</td>
<td>0.00</td>
</tr>
<tr>
<td>SKBR3</td>
<td>347.00</td>
<td>MYC</td>
<td>2801.00</td>
<td>8.07</td>
<td>0.00</td>
</tr>
<tr>
<td>BT474</td>
<td>879.00</td>
<td>ERBB2</td>
<td>9305.00</td>
<td>26.80</td>
<td>0.00</td>
</tr>
<tr>
<td>COLO 320</td>
<td>1206.00</td>
<td>MYC</td>
<td>42076.00</td>
<td>34.90</td>
<td>0.00</td>
</tr>
<tr>
<td>UACC1598</td>
<td>783.00</td>
<td>MYC</td>
<td>41648.00</td>
<td>53.20</td>
<td>0.00</td>
</tr>
</tbody>
</table>

\( p^a \) was calculated from the discordance T test (see “Materials and Methods”).

Fig. 2. Two examples of the genomic hybridization on cDNA arrays. A, high-level amplification of MYC in colon cancer cell line Colo320. Signal intensity of MYC is 16-fold higher than the nonamplified signal intensity in this hybridization. B, hybridization of breast cancer cell line SKBR3. In this hybridization, the signal intensity ratios were 9-fold for MYC and 22-fold for ERBB2. The level of ERBB2 amplification has been determined to be 6-fold and MYC amplification 32-fold by interphase FISH. The grid indicates the pattern of cDNAs printed on the microarray.
and overexpression of ZNF133 have not been reported previously, but the amplification of this chromosomal region has been detected by CGH in several different types of malignancies. In a CGH study of 58 primary gastric cancers, 20p gain was observed in 38% of cases (16). In chondrosarcomas, gain of 20p was observed in 31% of the analyzed primary gastric cancers, 20p gain was detected in 38% of cases (16). CGH in several different types of malignancies. In a CGH study of 58 primary gastric cancers, larger arrays will provide an amplicon map at and genetic composition of the 20p amplicon from the small microarray used in this study, larger arrays will provide an amplicon map at higher density. This information should prove extremely useful for focusing efforts to identify amplification target genes. With sufficient cDNA density, it should prove possible to map core regions of amplification in multiple tumor specimens. This information can then be further correlated with expression patterns determined across the amplified region. When integrated with the rapidly emerging human genome sequence, this approach should greatly accelerate the discovery of genes amplified during tumor progression.

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References

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