CGH, cDNA and tissue microarray analyses implicate *FGFR2* amplification in a small subset of breast tumors

Mervi Heiskanen [a,*], Juha Kononen [a], Maarit Bärlund [b], Joachim Torhorst [c], Guido Sauter [c], Anne Kallioniemi [a] and Olli Kallioniemi [a]

[a] *Cancer Genetics Branch, National Human Genome Research Institute, National Institutes of Health, 49 Convent Drive MSC 4470, Room 4A15, Bethesda, MD 20892-4470, USA*

[b] *Laboratory of Cancer Genetics, Institute of Medical Technology, University of Tampere and Tampere University Hospital, 33521 Tampere, Finland*

[c] *Institute of Pathology, University of Basel, Switzerland*

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Multiple regions of the genome are often amplified during breast cancer development and progression, as evidenced in a number of published studies by comparative genomic hybridization (CGH). However, only relatively few target genes for such amplifications have been identified. Here, we indicate how small-scale commercially available cDNA and CGH microarray formats combined with the tissue microarray technology enable rapid identification of putative amplification target genes as well as analysis of their clinical significance. According to CGH, the SUM-52 breast cancer cell line harbors several high-level DNA amplification sites, including the 10q26 chromosomal region where the fibroblast growth factor receptor 2 (*FGFR2*) gene has been localized. High level amplification of *FGFR2* in SUM-52 was identified using CGH analysis on a microarray of BAC clones. A cDNA microarray survey of 588 genes showed >40-fold overexpression of *FGFR2*. Finally, a tissue microarray based FISH analysis of 750 uncultured primary breast cancers demonstrated *in vivo* amplification of the *FGFR2* gene in about 1% of the tumors. In conclusion, three consecutive microarray (CGH, cDNA and tissue) experiments revealed high-level amplification and overexpression of the *FGFR2* in a breast cancer cell line, but only a low frequency of involvement in primary breast tumors. Applied to a genomic scale with larger arrays, this strategy should facilitate identification of the most important target genes for cytogenetic rearrangements, such as DNA amplification sites detected by conventional CGH.

Figures on http://www.esacp.org/acp/2001/22-4/heiskanen.htm.

1. Introduction

Cytogenetic and molecular cytogenetic studies have revealed a tremendous complexity of chromosomal rearrangements in cancer, particularly in common solid tumor types, such as breast cancer. For example, CGH studies have produced a detailed map of specific chromosomal sites were DNA amplifications take place in cancer ([18], http://www.helsinki.fi/~lgl_www/CMG2.html). However, the molecular consequences of these cytogenetic rearrangements are poorly understood, as only a small fraction of the genes involved in such rearrangements are currently known. For example, in breast cancer, more than 30 DNA amplification sites have been identified, of which only a small portion harbor oncogenes known to be involved in breast cancer development or progression ([12, 16], http://www.nhgri.nih.gov/DIR/CGB/CR2000/). In a few cases, identification of candidate target genes for such novel DNA amplifications has been successful. These include the *ZNF217*, *Aurora II* and *AIB1* genes at 20q [3,4,8] as well as *PIK3CA* at 3q [27], and *RPS6KB1* at 17q23 [7,9].

CGH and other cytogenetic and molecular cytogenetic techniques have an inherently low resolution and the rearranged chromosomal regions are also often very large and complex. Therefore, identification of amplification target genes using positional

*Corresponding author: Mervi Heiskanen, Ph.D. Current affiliation: Compugen Inc., 9700 Great Seneca Hwy, Rockville, MD 20850, USA. Tel.: +1 240 402 3241; Fax: +1 301 340 0033; E-mail: mervi@cgen.com.

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cloning techniques remains very tedious and the analysis of multiple loci throughout the genome is virtually impractical with classical techniques. Several high-throughput microarray technologies have been developed for large-scale genomic screens. cDNA microarrays [15,26] allow screening for expression levels of thousands of genes simultaneously. High-density arrays of genomic or cDNA clones can be used for copy number analysis of multiple loci or genes across the genome at once [14,23,24,28]. These techniques have a much higher resolution than conventional CGH and therefore should facilitate the identification of the target genes for cytogenetic rearrangements.

Finally, identification of cancer genes is often accomplished in cell lines and other model systems, and it remains important to validate that these alterations also take place *in vivo*. Tissue microarrays that contain hundreds of primary tumors on a single microscope slide substantially facilitate the study of the frequency and clinical significance of genetic alterations identified in model systems [19].

Here, we present a strategy where a combination of these microarray technologies was applied for screening of amplified and overexpressed genes in the SUM-52 breast cancer cell line. The SUM-52 cell line was selected for this study because it contains several high-level amplification sites detected by CGH ([11], http://www.nhgri.nih.gov/DIR/CGB/CR2000/). In the second phase, a tissue microarray analysis of 750 primary breast cancers was performed to evaluate the *in vivo* prevalence of the alterations found in this cell line model system.

2. Materials and methods

2.1. Genomic microarrays

Genomic microarrays (gCGH, GenoSensor^{TM}, Vysis Inc., Downers Grove, IL) used in this study were composed of BAC clones representing 31 different genomic regions previously reported to be amplified in human cancers. Each locus was arrayed in five replicates on a 14×9 mm area. gCGH analysis was done according to manufacture's instructions (Vysis). Briefly, genomic DNA from the SUM-52 breast cancer cell line was labeled with SpetrumGreen using nick translation (Microarray Nicktranslation Kit, Vysis). SpectrumRed labeled normal DNA was provided by Vysis. 250 ng of labeled SUM-52 DNA was hybridized together with normal reference DNA overnight at 37°C. Repetitive sequences were blocked with Cot-1 DNA (Vysis). After washing and counterstaining with DAPI, hybridization signals were imaged using a multicolor large field fluorescence imaging system (GenoSensor Reader System, Vysis).

2.2. cDNA microarrays

Gene expression analysis was done using a membrane-based cDNA microarray containing 588 clones spotted in duplicates (Atlas cDNA array, Clontech, Palo Alto, CA). Total RNA from SUM-52 cell line was extracted using the RNeasy kit (Qiagen, Valencia, CA) and mRNA was purified using poly-dT coated magnetic-beads (Dynabeads, Dynal, Oslo, Norway). Reference mRNA was isolated in a same manner from normal breast total RNA (Clontech). One μg of mRNA from both normal breast tissue and SUM-52 cell line was radioactively labeled with ^{32}P dCTP using a single-pass first strand cDNA synthesis. The probes were purified by gel-filtration chromatography (Bio-Rad, Hercules, CA) and remaining RNA was degraded by alkaline hydrolysis, followed by neutralization of the cDNA. The membrane was prehybridized for one hour at 42°C in ExpressHyb solution (Clontech). All of the synthesized labeled cDNAs were hybridized on two identical membranes overnight at 42°C. The membranes were washed 4 times in $2 \times$ SSC/0.1% SDS and two times in $0.2 \times$ SSC/0.1% SDS at 68°C to remove unbound probe. The membranes were exposed to phosphorimager plates for 24 h, and scanned with 50 μm resolution. Intensity ratios were calculated for each of the double-spots on the membranes using ImageQuant software (Molecular Dynamics, Sunnyvale, CA).

2.3. Tissue microarrays

Two tissue microarrays were used in this study. The first array consisted of 372 ethanol-fixed breast cancers [19]. The second microarray consisted of 612 formalin-fixed paraffin-embedded primary breast cancers from the years 1985–1995, from patients with complete clinico-pathological information including an average of 5.4 years of follow-up. Both series of tumors were analyzed, with 750 cases being informative. Both tumor sets were obtained from the Institute of Pathology, University of Basel. The tumor samples were reviewed by one pathologist (J.T.) and included 73.3% ductal, 13.6% lobular, 3% medullary, 2.6% mucinous, 1.5% cribriform, 1.4% tubular, 1.1% papillary carcinomas, 1.9% ductal carcinoma *in situ*, and 1.7%

of other rare histological subtypes. The grade distribution was 24% grade 1, 40% grade 2, and 36% grade 3. The pT stage was pT1 in 32%, pT2 in 51%, pT3 in 7%, and pT4 in 10%. The average age of the patients was 60 years (range 26–97 years), with 50% having a node-negative and 50% a node-positive disease. All specimens evaluated were anonymous, archival tissue specimens.

2.4. Fluorescence in situ hybridization

Tissue microarray FISH was performed as described earlier [6,19]. Briefly, sections were deparaffinized, permeabilized, deproteinated, air dried and dehydrated in 70, 85 and 100% ethanol followed by denaturation for 5 minutes at 74°C in 70% formamide/2 × SSC solution. A BAC clone specific for the *FGFR2* gene (Vysis) was labeled with SpetrumOrange (Vysis) by random priming (BioPrime DNA labeling kit, GibcoBRL). The hybridization mixture contained 30 ng of each of the probes and 15 μg of human Cot-1 DNA. After overnight hybridization at 37°C, slides were washed and counterstained with 0.2 μM DAPI in an antifade solution. FISH signals were evaluated using a Zeiss fluorescence microscope and over 10 FISH signals per cell or tight clusters of signals were considered as criteria for gene amplification.

3. Results

Figure on http://www.esacp.org/acp/2001/22-4/heiskanen.htm.

We first hybrizided genomic DNA extracted from SUM-52 breast cancer cell line on gCGH microarray for detection of gene amplifications. The tumor to normal (green/red) ratio of the *FGFR2* gene was 16.2, indicating high level amplification of this gene in the SUM-52 cell line (Fig. 1a). This result fits well with metaphase CGH data showing high level amplification of 10q26 in SUM-52 (Fig. 1b) [11]. The amplification of *FGFR2* was confirmed using interphase FISH which showed more than 40 copies of the *FGFR2* gene per cell (Fig. 1c).

In order to evaluate gene expression changes, mRNA from the SUM-52 cell line was used in radioactive cDNA microarray analysis of 588 genes. The most dramatic expression ratio difference was an over 40-fold

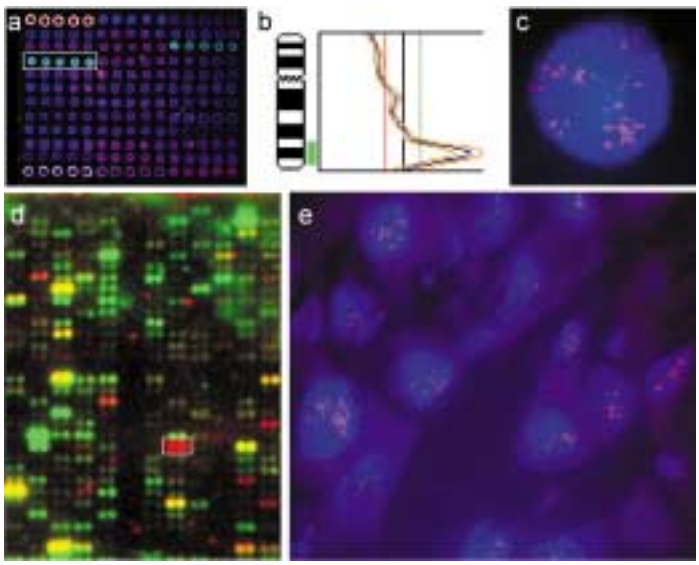

Fig. 1. Identification of *FGFR2* amplification in breast cancer by DNA and tissue microarray technologies. (a) Hybridization of SUM-52 genomic DNA (green) together with normal reference DNA (red) on a gCGH microarray containing 31 BAC clones in five replicates each. The *FGFR2* gene (boxed) showed an average red-to-green ratio of 16 indicating high level amplification of this gene. (b) High-level amplification of 10q26 chromosomal region in SUM-52 breast cancer cell line by chromosome CGH. (c) *In situ* hybridization with *FGFR2* BAC clone confirms high level amplification of FGFR2 in SUM-52 breast cancer cell line. (d) Expression screening of 588 genes in SUM-52 cells using radioactive cDNA microarrays. Hybridization of SUM-52 cDNA is shown in pseudocolor red and normal reference cDNA in pseudocolor green. The red-to-green ratio of 41 for *FGFR2* (boxed) indictates that *FGFR2* is highly overexpressed. (e) Analysis of copy number levels of *FGFR2* in 750 primary breast tumors using FISH to tissue microarrays showed amplification in about 1% of the tumors. A representative tumor sample with high level amplification is shown. This figure can be viewed on http://www.esacp.org/acp/2001/22-4/heiskanen.htm.

upregulation of the *FGFR2* gene (Fig. 1d). Subsequent Northern blot analysis confirmed the high-level overexpression of *FGFR2* [29]. The high-level amplification and massive overexpression implicated *FGFR2* as a putative target gene of the 10q26 amplification in the SUM-52 cells.

The prevalence of *FGFR2* amplification *in vivo* in uncultured primary breast cancers was evaluated using FISH analysis on tissue microarrays. Here, we used two tissue microarrays containing 372 and 612 specimens. Altogether, 750 tumors were informative. Only seven ($\sim$1%) tumors contained high-level amplification of the *FGFR2* gene (Fig. 1e).

4. Discussion

Identification of DNA amplification target genes may pinpoint genes with a critical role in cancer development and progression. There are over 30 different chromosomal regions that often undergo amplification in breast cancer ([10–12], http://www.nhgri.nih.gov/DIR/CGB/CR2000/). Only few of the amplification target genes have been identified, and even fewer have been shown to correlate with prognosis or other clinical characteristics of the cancer patients. One of these genes, *HER-2*, is now used as an immunotherapeutic target for breast cancer using the Herceptin drug [21]. It took 15 years from the identification of the *HER-2* gene to studies demonstrating the clinical utility of anti-HER-2 therapy [25]. This demonstrates the slow process of translating gene discoveries to the clinical situation.

The sequencing of the human genome is now being completed and in principle it is possible to print the whole representation of human genome on microarrays. cDNA microarray analyses have already demonstrated how cancer is characterized by abnormal expression of hundreds or thousands of different genes [2,5,13,17,22]. A small fraction of these gene expression changes are likely to be caused by genomic rearrangements, including amplifications, translocations and specific rearrangements. The significance of these genomic alterations is that genes implicated in them are more likely to be primary alterations that directly contribute to the clonal progression of cancer than genes that are merely showing differential expression. Therefore, CGH microarrays and copy number analyses may provide additional value to large-scale gene expression surveys. Although *FGFR2* has been previously implicated in breast cancer [1,20] larger scale application of the CGH microarray technology will have the potential to identify similar target genes for genomic alterations affecting other chromosomal regions in cancer.

In vivo validation of results obtained from experimental model systems is essential. Tissue microarrays provide a quick and highly effective method for this purpose as this technology enables analysis of hundreds of clinical specimens at once, and substantially facilitate translation of molecular findings to clinical applications. In this study, *FGFR2* turned out to be very infrequently amplified in breast cancer according to a large-scale tissue microarray analysis. The frequency of *FGFR2* involvement in previous studies has been higher, ranging from 4% to 11.5% [1,20]. It remains to be determined, whether this is due to the selection of the patient populations. DNA and RNA for molecular analyses (Southern blot and Northern blot) can usually only be acquired from large, clinically more aggressive tumors. In contrast, tissue microarray analyses require minimal amount of tissue, allowing the sampling of tumors in a more unbiased manner.

In summary, genomic microarrays and cDNA microarrays were used to identify *FGFR2* as a putative target gene for 10q26 amplification in the SUM-52 breast cancer cell line. Tissue microarray analysis showed that *FGFR2* is only amplified in 1% of breast cancers. This study illustrates how the combined use of three microarray techniques provides a powerful strategy for the identification of amplification target genes in cancer.

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