Tissue microarrays for high-throughput molecular profiling of tumor specimens

JUHA KONONEN[1], LUKAS BUBENDORF[2], ANNE KALLIONIEMI[1], MAARIT BARLUND[3], PETER SCHRAML[2], STEPHEN LEIGHTON[4], JOACHIM TORHORST[2], MICHAEL J MIHATSCH[2], GUIDO SAUTER[2] & OLLI-P. KALLIONIEMI[1]

[1]*Laboratory of Cancer Genetics, National Human Genome Research Institute, National Institutes of Health, 49 Convent Drive MSC 4470, Room 4A24, Bethesda, MD 20892-4470, USA*
[2]*Institute of Pathology, University of Basel, Switzerland*
[3]*Laboratory of Cancer Genetics, Institute of Medical Technology, University of Tampere and Tampere University Hospital, P.O. Box 2000, FIN-33521 Tampere, Finland*
[4]*Beecher Instruments, Silver Spring, MD, USA*
Correspondence should be addressed to O-P.K; email: okalli@nhgri.nih.gov

Many genes and signalling pathways controlling cell proliferation, death and differentiation, as well as genomic integrity, are involved in cancer development. New techniques, such as serial analysis of gene expression and cDNA microarrays, have enabled measurement of the expression of thousands of genes in a single experiment, revealing many new, potentially important cancer genes[1,2]. These genome screening tools can comprehensively survey one tumor at a time; however, analysis of hundreds of specimens from patients in different stages of disease is needed to establish the diagnostic, prognostic and therapeutic importance of each of the emerging cancer gene candidates. Here we have developed an array-based high-throughput technique that facilitates gene expression and copy number surveys of very large numbers of tumors. As many as 1000 cylindrical tissue biopsies from individual tumors can be distributed in a single tumor tissue microarray. Sections of the microarray provide targets for parallel *in situ* detection of DNA, RNA and protein targets in each specimen on the array, and consecutive sections allow the rapid analysis of hundreds of molecular markers in the same set of specimens. Our detection of six gene amplifications as well as p53 and estrogen receptor expression in breast cancer demonstrates the power of this technique for defining new subgroups of tumors.

Original tissue sample sources are morphologically representative regions of regular formalin-fixed paraffin-embedded tumor blocks, or from freshly frozen tumors fixed in cold ethanol and embedded in paraffin to preserve intact RNA and DNA in each tissue specimen (Figs. 1 and 2). Core tissue biopsies (diameter, 0.6 mm; height, 3–4 mm) are taken from individual 'donor' paraffin-embedded tumor blocks and precisely arrayed into a new 'recipient' paraffin block (45 × 20 mm) (Fig. 1) using a custom-built instrument. We prefer 0.6 mm cylinders, which preserve histological information while allowing as many as 1000 specimens to be arrayed in each recipient block with minimal damage to the original blocks. Automation would permit simultaneous construction of multiple tumor array blocks with identical sample coordinates. Sections cut from the array allow parallel detection of DNA (fluorescence *in situ* hybridization, FISH), RNA (mRNA ISH) or protein (immunohistochemistry, IHC) targets in each of the hundreds of specimens in the array. At least 200 consecutive sections 4–8 μm in thickness can be cut from each tumor array block. This allows consecutive analyses of a large number of molecular markers and construction of a database of correlated genotypic or phenotypic characteristics of uncultured human tumors.

We tested the potential of this array technology by assembling two breast cancer tissue array replicas, each with 645 samples. Cold ethanol fixation was used in 372 of the specimens to preserve high-molecular-weight DNA and RNA (Fig 2). Tissues fixed in a uniform manner provide a substantial advantage compared with variably fixed archival tissues for analyses based on *in situ* hybridization. We first used sections of this array in FISH analyses of gene amplifications in breast cancer. FISH is an excellent method for visualization and accurate detection of genetic rearrangements (amplifications, deletions or translocations) in individual, morphologically defined cells[3–5]. The utility of FISH in large-scale studies of clinical specimens is limited by the requirement to hybridize, stain and interpret tumor specimens one slide at a time. However, when combined with the tumor array technology, FISH becomes a powerful high-throughput method allowing the analysis of hundreds of specimens per day. To demonstrate this, we analyzed the amplification of six loci and genes in the 372 ethanol-fixed, arrayed primary breast cancer specimens. In just a few days, frequencies of amplification were determined for the three major breast cancer oncogenes, *ERBB2* (18%), *MYC* (25%) and *CCND1* (24%). These frequencies agree with published results[3,5–8]. Two recently discovered regions of DNA amplification in breast cancer, 17q23 and 20q13 (refs. 9–11), were also analyzed, using probes from the cores of contigs developed for positional cloning of the putative amplification target genes at these chromosomal regions[11,12]. High-level amplification of 17q23 was found in 13% and that of 20q13 in 6% of the tumors. The oncogene *MYBL2*, recently localized to 20q13.1 (ref. 13), and overexpressed in breast cancer cell lines compared with normal breast tissue according to cDNA array hybridization (data not shown), was found to be amplified in 7% of the same set of tumors. *MYBL2* was amplified in tumors with a normal copy number of the main 20q13 locus, indicating that it may define an independently selected region of amplification at 20q. Amplification of at least one of the six loci was detected in 52% of primary tumors (Fig. 3).

Consecutive sections from the arrays allow different DNA, RNA or protein targets to be analyzed from defined, morpholog-

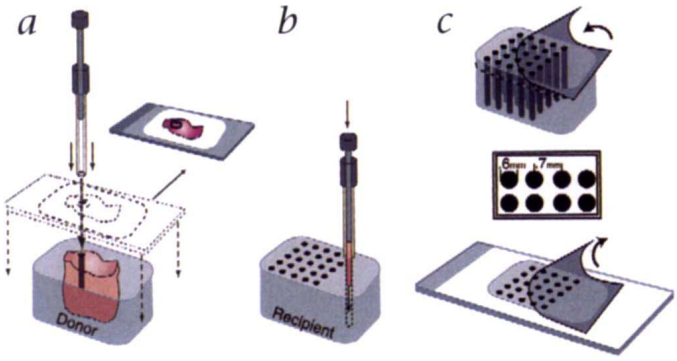

Fig. 1 Tumor array construction. ***a***, Small tissue core biopsies are punched from selected regions of donor blocks using a thin-wall stainless steel tube sharpened like a cork borer. HE-stained sections overlayed on the surface of the donor blocks guide sampling from morphologically representative sites in the tumors. ***b***, A solid stainless steel stylet transfers tissue cores into defined array coordinates in the recipient block. ***c***, An adhesive-coated tape sectioning system assists in cutting the tissue microarray block. Each tissue element in the array is 0.6 mm in diameter and spacing between two adjacent array elements is 0.1 mm.

ically almost identical regions of the tumors (Fig. 2). Analyses of the breast cancer array by mRNA ISH and IHC indicated that all *ERBB2*-amplified tumors overexpressed the *ERBB2* mRNA and protein, as expected for an amplification target gene. Moreover, the estrogen receptor (ER) and p53 data allowed stratification of the tumors in four different subgroups, each with a different pattern of clinical features and gene amplifications (Fig. 3). Thus, the co-amplification patterns of the six loci analyzed by FISH were grouped according to tumor ER and p53 expression (Fig. 3*c* and *d*). Our data confirmed many of the clinicopathological correlations of gene amplifications reported with conventional techniques based on DNA isolated from entire tumors. For example, *ERBB2* and *MYC* were amplified more often in tumors lacking steroid receptors (P = 0.004 and P = 0.03, respectively) whereas the opposite was observed for *CCND1* (P = 0.12) (refs. 14,15). Furthermore, tumors with p53 immunostaining had a particularly high frequency of *MYC* ($P < 0.0001$) and *ERBB2* (P = 0.028) amplifications ,compared with $p53^-$ tumors. These combined data from gene amplification and expression analyses illustrate the power of the tumor array technology in rapid, comprehensive somatic genetic and phenotypic characterization of a large panel of uncultured human tumors.

Because most tumors are heterogeneous, very small tissue samples may not always reflect the biological properties of the entire tumor. However, sampling from morphologically defined regions permits targeting of the most representative (for example, poorly differentiated) areas of each tumor. We found that analyses of the ER content from a tissue homogenate by biochemical means and by IHC from tumor tissue arrays had 84% concordance (n = 210), which is in the range reported in comparisons of biochemical and conventional whole-section IHC ER analysis[16,17]. The prevalence of gene amplifications and their association with other clinical and molecular parameters reported here are consistent with previous results[15]. Furthermore, *ERBB2* FISH data obtained from tumor array sections and disaggregated nuclei from whole mount sections were in perfect agreement in all 14 cases tested. However, accurate sampling from histologically representative regions of each tumor is important and may sometimes warrant collection of multiple samples from different sites in each tumor. Finally, tumor tissue microarrays probably will be most useful for the initial screening of large numbers of tumors for clinical research purposes, and the results may need to be verified by analyses of larger tissue specimens before clinical application. This is especially pertinent for tumors with very prominent intra-tumor heterogeneity.

At present, 1000 tumors can be arrayed in one 45 × 20 mm block. Smaller cylinders, as well as arraying at a higher density or on a larger recipient block, could provide a much higher number of specimens per array. Thus, the tumor array technology provides an increase of an order of magnitude in the number of specimens available for analysis, compared with multi-tissue blocks', in which a few dozen individual formalin-fixed specimens are arranged in a less defined configuration and used for antibody testing[18–20]. Other advantages of our approach include minimal destruction of the original tissue blocks as well as expansion of the utility of this technique to the visualization of DNA and RNA targets. The tissue array technology facilitates procurement, distribution and application of human tumor tissues for research purposes. Automation of the procedure for sampling and arraying would allow the creation of dozens of replicas of tumor arrays, each providing up to 200 sections for molecular analyses. In prin-

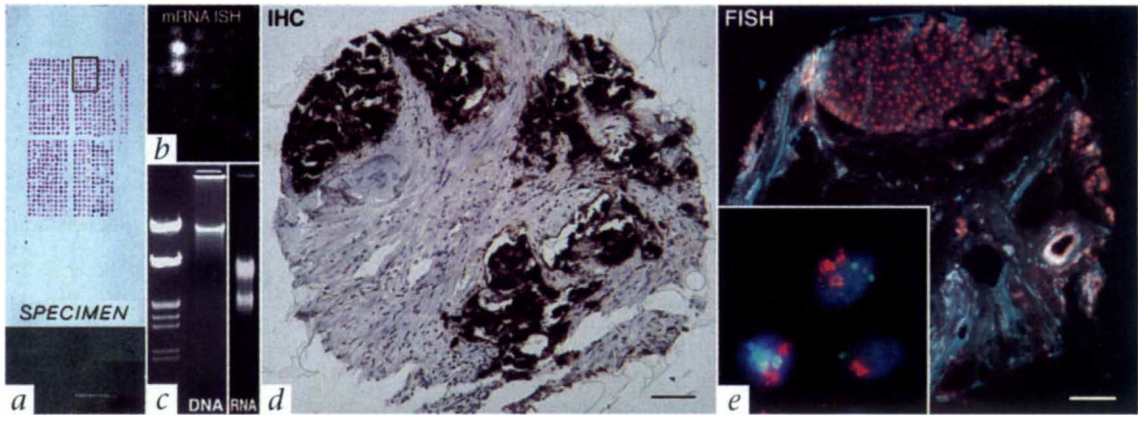

Fig. 2 ***a***, HE-stained tumor tissue microarray slide. Specimens were arranged in five subdivisions to assist interpretation of the histochemical analyses. ***b***, Magnification of an autoradiogram of *ERBB2* mRNA in situ hybridization on tissue array from the region in the small rectangle in *a*. Two adjacent specimens in the array show strong hybridization signals. ***c***, DNA and RNA gels. High-molecular weight DNA and RNA can be extracted using standard methods from breast cancer specimens fixed in ethanol at 4 °C overnight. Left lane of DNA gel, lambda-DNA molecular weight marker. ***d***, Immunocytochemical detection of *ERBB2* overexpression in one tissue array element. ***e***, High-level *ERBB2* gene amplification detected by FISH in one tumor array element. The inset shows three nuclei, each with many tightly clustered red *ERBB2* hybridization signals and two copies of the green centromeric reference probe. Not all signals are in focus at the same time. Bar in *d* and *e*, 80 μm.

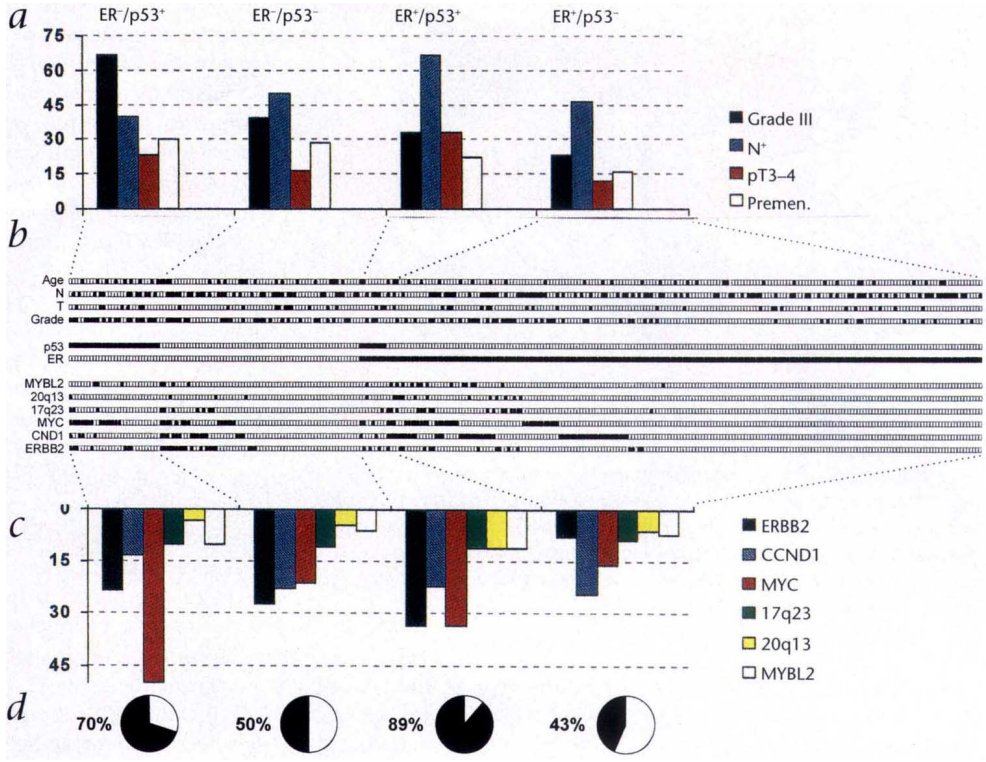

Fig. 3 Summary of results obtained with the tumor tissue microarray technology in the characterization of ethanol-fixed breast cancer specimens by immunohistochemistry and by fluorescence in situ hybridization. Tumors were classified by estrogen receptor (ER) content and p53 immunostaining. Only tumors with complete data were included. ***a***, Distribution of clinical features of the tumors in the four groups. The percentage of grade III tumors, lymph-node positive disease (N^+), tumors with a pT class 3 or 4 (pT3–4), and patients with age < 50 years (Premen.) are shown for each group. ***b***, A schematic binary data format arrangement of all the tumors showing clinical features (top four rows contain information originating from patient records, shown as a reference only), immunohistochemistry results (middle two rows) and DNA amplification status (bottom six rows). Each square represents one data point for one tumor, and one column of squares provides all the properties of a given tumor. Tumors were arranged by ER and p53 status. Filled squares: Top, unfavorable clinical features (young age, node-positivity (N), large tumor size (T), or grade III); middle, immunopositivity (with ER and p53 antibodies); bottom, the presence of gene amplification (for each of the 6 genes/loci tested). ***c***, The percentage of DNA amplifications at the 6 different loci tested by FISH in the four subgroups of tumors. ***d***, The overall pe centage of tumors in each ER/p53 phenotypic subgroup with amplification the six loci.

ciple, entire archives of tens of thousands of existing formalin-fixed tissues from pathology laboratories could be placed in a few dozen high-density tissue microarrays to survey a variety of tumor types as well as different stages of tumor progression. The tumor array strategy would allow hundreds of potential prognostic or diagnostic molecular markers to be tested.

We have developed a research tool for high-throughput, molecular characterization of large panels of uncultured tumor specimens in a tissue array format. The tissue array technique has been adapted as a routine tool in our laboratory for molecular analyses of the DNA, RNA and protein targets in large series of tumors. For example, we have constructed an array of 450 prostate cancer specimens, as well as a multi-tumor array of 350 specimens representing various types of tumors. This technology will probably have many applications in fields other than cancer research. For example, tissue microarray analyses of the expression of multiple genes could be applied to the study of other diseases and to normal human or animal tissues, including repositories of tissues from transgenic animals or cultured cells.

Methods

Material. Samples (645 total) were arranged in five separate subdivisions to assist interpretation of the analyses of the breast tumor tissue microarrays. These included 372 ethanol-fixed tumors and 132 archival formalin-fixed breast cancers. The control section of the array included 52 formalin-fixed normal tissues and 89 fixation controls (large specimens of breast cancer divided in pieces and fixed with ethanol or formalin for 12 hours at room temperature, 4 °C or −20 °C). Breast cancer samples for ethanol fixation were selected at random from the fresh-frozen tumor bank of the Institute of Pathology, University of Basel. Using the fixation control specimens, we discovered that both formalin-fixed and ethanol-fixed tissues are suitable for FISH analyses, but that the quality of FISH experiments is more uniform and reliable in ethanol-fixed tissues than in formalin-fixed tissues. Therefor in this series, only data from those 372 tumors were used for gene amplif cation analyses. This subset was reviewed by one pathologist. The series ir cluded 259 ductal, 52 lobular, 9 medullary, 6 mucinous, 3 cribriform, tubular, 2 papillary, 1 histiocytic, 1 clear cell, and 1 lipid rich carcinom There were also 15 ductal carcinomas *in situ* and 2 carcinosarcomas. Th following were not otherwise classified: 4 primary carcinomas that ha been treated with chemotherapy before surgery, 8 recurrent tumors and metastases. Of the remaining tumors, 24% were grade 1, 40% grade 2, an 36% grade 3. The pT stage was pT1 in 29%, pT2 in 54%, pT3 in 9%, an pT4 in 8%. Axillary lymph nodes had been examined in 282 patients (45 pN0, 46% pN1, 9% pN2).

Construction of tumor tissue microarrays. An instrument was built f creating holes in the recipient array blocks and for acquiring tissue cor from the donor blocks. The instrument consisted of a thin wall stainless ste tube with an inner diameter of about 600 µm and sharpened like a co borer (the 'needle'), held in an X–Y precision guide. A solid stainless ste wire closely fit in the tube acted as a stylet to empty the tube contents or t transfer tissue cores into the recipient block. The precision guide allowe the needle to move only parallel to its own axis. This guide was in tur mounted vertically on a horizontal xy stage and moved with digital m crometers to successive locations in the growing array. The donor block w manually positioned for sampling based on a visual alignment with the co responding HE-stained section on a slide. The slide was removed before th block surface was punched. To best select the areas of interest, we use stereotactic microscope, with an additional bright light source positione under each block to provide more contrast than surface illumination of th blocks. An adjustable depth-stop maintained the length of the recipie holes and the transferred cores. The needle was used to retrieve a cylindric sample from a selected region in the donor block and to extrude the samp core directly into the recipient block with defined array coordinates. Th sampling cycle was repeated to punch different regions in the same don block and/or hundreds of different donor blocks. As many as 1000 such ti sue cylinders could be placed in one 45 × 20 mm recipient paraffin bloc

After the block construction was completed, 8-μm sections of the resulting tumor tissue microarray block were cut with a microtome. Melting of the tumor array block surface can be used to ensure easier sectioning, but this was avoided as it might produce moving of tissue cylinders in the block. An adhesive-coated tape system (Instrumedics, Hackensack, New Jersey) was a useful method for sectioning the tumor array blocks. The microtome knife cuts underneath a piece of tape that is placed over the block surface. The thin tissue section adheres to the tape, which is then rolled on an adhesive-coated microscope slide to transfer the section to the slide. On average, 200 sections can be cut from one tumor tissue microarray block. HE-staining for histology verification was done on every 40th section cut from the block. Nearly all of the malignant tumors retain their histological pattern through the entire 3-mm-deep block. Some well-differentiated cancers or other smaller lesions, as well as normal glandular tissues, retained their morphology for a few dozen sections (usually to the depth of 1–2 mm).

Immunohistochemistry. Standard indirect immunoperoxidase procedures were used for immunohistochemistry (ABC-Elite, Vector Laboratories). Monoclonal antibodies from DAKO (Glostrup, Denmark) were used for detection of p53 (DO-7, mouse, 1:200 dilution), ERBB2 (c-erbB-2, rabbit, 1:4000), and estrogen receptor (ER ID5, mouse, 1:400). Microwave pretreatment was used for p53 (30 minutes at 90 °C) and ERBB2 antigen (60 minutes at 90 °C) retrieval. Diaminobenzidine was used as a chromogen. Tumors with known positivity were used as positive controls. The primary antibody was omitted for negative controls. Tumors were considered positive for ER and p53 if an unequivocal nuclear positivity was seen in at least 10% of tumor cells. The ERBB2 staining was subjectively graded into 2 groups: negative (no staining or weak membranous positivity) and strongly positive (strong membranous positivity in at least 10 % of tumor cells). One 645-specimen array slide can be scanned and scored with a ×10 objective in less than 2 hours (including data recording).

Fluorescent *in situ* hybridization. Two-color FISH was undertaken using Spectrum Orange-labeled *CCND1*, *MYC* or *ERBB2* probes with corresponding FITC-labeled centromeric reference probes (Vysis, Downer's Grove, Illinois). One-color FISH was done with Spectum Orange-labeled 20q13 minimal common region[12] (Vysis, Downer's Grove, Illinois), *MYBL2* and 17q23 probes[11]. Before hybridization, tumor array sections were deparaffinized, 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. The hybridization mixture contained 30 ng of each of the probes and 15 μg of human Cot1 DNA. After overnight hybridization at 37 °C in a humidified chamber, slides were washed and counterstained with 0.2 μM DAPI in an antifade solution. FISH signals were scored with a Zeiss fluorescence microscope equipped with double-band pass filters for simultaneous visualization of FITC and Spectrum Orange signals using ×40 to ×100 objectives. Greater than 10 FISH signals per cell or tight clusters of signals in > 5% of cells in confined region(s) of the sections were the criteria for gene amplification.

mRNA *in situ* hybridization. Tumor array sections were deparaffinized and air dried before hybridization. Synthetic oligonucleotide probe directed against *ERBB2* mRNA (Genbank accession number X03363; nucleotides 350–396) was labeled at the 3′-end with ^{33}P-dATP using terminal deoxynucleotidyl transferase. Sections were hybridized in a humidified chamber at 42 °C for 18 hours with 1×10^7 CPM/ml of the probe in 100 μl of hybridization mixture (50% formamide, 10% dextran sulphate, 1% sarkosyl, 0.02 M sodium phosphate, pH 7.0, 4× SSC, 1× Denhardt's solution and 10 mg/ml ssDNA). After hybridization, sections were washed several times in 1× SSC at 55 °C to remove unbound probe, and briefly dehydrated. Sections were exposed for three days to phosphorimager screens to visualize *ERBB2* mRNA expression. Sections treated with RNase before hybridization were used as negative controls.

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