Detecting Activation of Ribosomal Protein S6 Kinase by Complementary DNA and Tissue Microarray Analysis

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Background: **Studies by comparative genomic hybridization (CGH) have shown that chromosomal region 17q23 is amplified in up to 20% of primary breast cancers. We used microarray analyses to measure the expression levels of genes in this region and to explore their prognostic importance.** ***Methods:*** **A microarray that contained 4209 complementary DNA (cDNA) clones was used to identify genes that are overexpressed in the MCF-7 breast cancer cell line as compared with normal mammary tissue. Fluorescence *in situ* hybridization was used to analyze the copy number of one overexpressed gene, ribosomal protein S6 kinase (S6K), and to localize it to the 17q23 region. Northern and western blot analyses were used to measure S6K gene and protein expression, and an enzymatic assay was used to measure S6K activity. Tumor tissue microarray analysis was used to study amplification of S6K and the HER-2 oncogene, another 17q-linked gene, and the relationship between amplification and prognosis was analyzed. The Kaplan–Meier method was used for data analysis, and the log-rank test was used for statistical analysis. All *P* values are two-sided.** ***Results:*** **S6K was amplified and highly overexpressed in MCF-7 cells relative to normal mammary epithelium, and protein expression and enzyme activity were increased. S6K was amplified in 59 (8.8%) of 668 primary breast tumors, and a statistically significant association between amplification and poor prognosis (*P* = .0021) was observed. Amplification of both S6K and HER-2 implied particularly poor survival (*P* = .0001).** ***Conclusions:*** **The combination of CGH information with cDNA and tissue microarray analyses can be used to identify amplified and overexpressed genes and to evaluate the clinical implications of such genes and genomic rearrangements. S6K is likely to be one of the genes at 17q23 that is amplified during oncogenesis and may adversely affect the prognosis of patients with this amplification.** **[J Natl Cancer Inst 2000;92:1252–9]**

Large-scale gene expression surveys, such as complementary DNA (cDNA) microarrays *(1),* serial analysis of gene expression *(2),* or high-throughput cDNA sequencing, have made it possible to screen for the expression levels of thousands of genes in a tumor. In a typical cDNA microarray experiment, up to 10 000 genes are simultaneously analyzed in a parallel fashion, revealing hundreds of genes that are differentially expressed relative to those of a control tissue. Analyzing and interpreting this genomic-scale information to pinpoint alterations in specific genes that could be utilized for developing clinical applications, such as finding targets with potential diagnostic, prognostic, or therapeutic applications, have become increasingly important challenges. To rank the large number of targets for further study, it is important to separate primary genetic alterations from secondary, downstream changes in gene expression.

Gene amplification is an important mechanism for increasing expression of critical genes involved in the initiation and progression of cancer. Indeed, in breast cancer, a number of important oncogenes have already been found to be activated by DNA amplification. These oncogenes include HER-2 (at chromosome 17q12), C-MYC (at 8q24), PRAD1/CYCLIN D (at 11q13), FGFR-1 (at 8p12), and FGFR-2 (at 10q24) *(3).* Furthermore, studies by comparative genomic hybridization (CGH) *(4–7)* and chromosome microdissection *(8)* have revealed that numerous other chromosomal regions frequently undergo amplification in breast cancer. Focused studies of the amplification region at 20q12–20q13 have implicated a number of overexpressed genes with putative oncogenic potential, such as AIB1, BTAK, CAS-1, and ZNF217 *(9–12),* indicating that multiple genes may be induced in a given amplification region.

Our laboratory is using cDNA microarray analyses of breast cancer cell lines that harbor high-level DNA amplifications to explore the consequences of these multiple genomic DNA amplifications on the pattern of gene expression. This strategy could highlight genes that are targets for the clonal selection process that leads to DNA amplification. Of particular interest are the consequences of genomic rearrangements at 17q23, a locus that has been shown by CGH to be amplified in up to 20% of primary breast cancers *(4–7).* We showed that at least two separate regions at 17q23 undergo high-level amplification in breast cancer *(13).*

Here we report the results of a cDNA microarray analysis of expression levels of 4209 clones in the MCF-7 breast cancer cell line. Tissue microarray *(14)* analysis of 668 primary breast cancers was used to determine the *in vivo* importance of the amplified and overexpressed genes that we discovered in the cell line and to explore the relationship between gene amplification and patient survival.

METHODS

Cell Lines

Breast cancer cell lines BT-474, HBL-100, MCF-7, MDA-436, and ZR-75-1wt were obtained from the American Type Culture Collection (Manassas, VA), KPL-1 from the German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany), and Sum-52 from S. P. Ethier (University of Michigan, Ann Arbor).

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See "Notes" following "References."

Construction of Primary Breast Tumor Microarrays

Two groups of primary breast cancer tissue specimens were obtained from the Institute of Pathology, University of Basel, Switzerland, for the construction of tissue microarrays. The first group consisted of 372 ethanol-fixed primary breast cancer specimens [described in *(14)*]. The second group consisted of 612 formalin-fixed, paraffin-embedded primary breast cancer specimens from the years 1985–1995 obtained from patients with clinicopathologic information that included an average of 5.4 years of follow-up. All specimens were anonymous, archival tissue specimens. The use of these specimens for retrospective analyses was approved by the Ethics Committee of the University of Basel, and their use for tissue microarray analysis was approved by the National Institutes of Health Institutional Review Board.

The tumor samples were reviewed by one pathologist (J. Torhorst) and included 73.3% ductal, 13.6% lobular, 3.0% medullary, 2.6% mucinous, 1.5% cribriform, 1.4% tubular, and 1.1% papillary carcinomas; 1.9% ductal carcinoma *in situ;* and 1.7% of other rare histologic subtypes. The grade distribution *(15)* was 24% grade 1, 40% grade 2, and 36% grade 3. The postoperative tumor–node–metastasis (pT) stage *(16)* was 32% pT1, 51% pT2, 7% pT3, and 10% pT4. The average age of the patients was 60 years (range = 26–97 years); 50% of these patients had lymph node-negative disease and 50% had lymph node-positive disease.

The tissue microarrays were constructed as described previously *(14)*. Briefly, a representative tumor area was selected from hematoxylin–eosin-stained sections of each tumor. The individual donor tissue blocks and the corresponding histologic slides were overlaid for tissue microarray sampling. A tissue microarray instrument (Beecher Instruments, Silver Spring, MD) was used to create holes in a recipient paraffin block, to obtain cylindrical core tissue biopsy samples with a diameter of 0.6 mm from the donor paraffin blocks, and to transfer these biopsy samples to the recipient block at defined array positions. Multiple 5-μm sections were cut from the tissue microarray block by use of a microtome with an adhesive-coated-tape sectioning system (Instrumedics, Inc., Hackensack, NJ).

CGH Analysis of MCF-7

CGH was done as described previously *(17)*. Briefly, 400 ng of MCF-7 genomic DNA was fluorescently labeled with SpectrumGreen–deoxyuridine triphosphate (dUTP) (Vysis, Inc., Downers Grove, IL) by the use of nick translation. Normal placental DNA was fluorescently labeled in the same manner with SpectrumOrange–dUTP (Vysis, Inc.). The labeled DNAs were hybridized to normal metaphase chromosomes, and the chromosomes were counterstained with 4′,6-diamidino-2-phenylindole (DAPI) (Boehringer Mannheim Biochemicals, Indianapolis, IN). The hybridizations were evaluated by use of a Zeiss microscope (Carl Zeiss, Inc., Thornwood, NY) and the Quips XL program (Vysis, Inc.). The fluorescence intensities of MCF-7 DNA and placental DNA were measured along chromosome 17, and their ratios (DNA sequence copy number in MCF-7 cells/copy number in normal tissue) were computed. The average copy number ratio from 10 chromosomes and the 95% confidence interval were calculated.

Construction of cDNA Microarray

A total of 4209 cDNA clones from the set of 15 289 IMAGE (i.e., Integrated Molecular Analysis of Genomes and Their Expression) consortium cDNA clones described earlier *(18–20)* were printed onto glass slides as described previously *(1)*. Radiation hybrid-mapping information was available for about 50% of them *(21)*. The set included 88 housekeeping genes that were used as controls in the normalization of the data and 135 clones from chromosome 17, including eight clones from 17q23. Based on the information in the radiation hybrid map *(21)*, this represents approximately 10% of all cDNAs from this region.

Fluorescent cDNA Microarray Analysis of MCF-7 Cells

Total RNA was extracted from MCF-7 cells by use of the RNeasy kit (Qiagen, Inc., Valencia, CA). The labeling and hybridization were done as described previously *(18,22)*. Briefly, 100–200 μg of total MCF-7 RNA was used as template to prepare cDNA probes labeled with dUTP coupled to Cyanine5 (Cy5) fluorescent dye (Amersham Pharmacia Biotech, Inc., Piscataway, NJ) by use of oligo(dT)-primed polymerization by SuperScript II reverse transcriptase (Life Technologies, Inc. [GIBCO BRL], Gaithersburg, MD). Probes complementary to normal mammary gland RNA (Clontech Laboratories, Inc., Palo Alto, CA) were prepared in the same manner, except that the dUTP was coupled to Cyanine3 (Cy3) fluorescent dye (Amersham Pharmacia Biotech, Inc.). The labeled cDNAs were combined with 8 μg of polydeoxyadenylic acid (Amersham Pharmacia Biotech, Inc.), 4 μg of *Escherichia coli* transfer RNA (Sigma Chemical Co., St. Louis, MO), and 10 μg Cot-1 DNA (Life Technologies, Inc.) in 0.15% sodium dodecyl sulfate (SDS) and 3× standard saline citrate (SSC) (i.e., 450 m*M* NaCl and 45 m*M* sodium citrate). The probe mixture was incubated at 98 °C for 2 minutes and at 4 °C for 10 seconds and was applied onto the microarray slide. Hybridization was carried out at 65 °C for 16 hours. The slides were washed in 0.5× SSC (i.e., 75 m*M* NaCl and 7.5 m*M* sodium citrate) and 0.01% SDS at 55 °C for 2 minutes each.

Analysis of cDNA Microarray Data

Fluorescence intensities at the targets were measured by use of a custom-designed laser confocal microscope equipped with a scanning stage, appropriate excitation and emission filters, and a photomultiplier tube detector *(18)*. Intensity data were integrated over 225-μm^2 pixels and recorded at 16 bits. The fluorescent images from the MCF-7 hybridization and from the hybridization with normal mammary tissue were scanned separately. The MCF-7 image was assigned a red color and the reference image was assigned a green color to form a pseudo-color image. In this image, spots that appear in red correspond to genes overexpressed in MCF-7 cells, and those that are green correspond to genes whose expression is lower in MCF-7 than in the control sample (normal mammary tissue).

The results of the cDNA microarray experiments were analyzed by use of the ArraySuite program (developed at the National Human Genome Research Institute, Bethesda, MD, by Y. Chen) based on the IPLab Spectrum platform *(23)*. The program identified and segmented spots in the images corresponding to the MCF-7 (red) and normal mammary (green) cDNA hybridizations. After background subtraction, average intensities at each spot in the MCF-7 hybridization were divided by the average intensity of the same spot in the normal mammary hybridization. The ratios were normalized on the basis of the distribution of ratios for the 88 housekeeping genes *(22)*. This ensured that the ratios obtained were not affected by differential labeling or hybridization efficiency of the two cDNAs, by the quantity of the labeled cDNA in the hybridization reaction, or by the photomultiplier voltage settings during scanning. The ArraySuite program also allows visualization of the image for each spot. For this study, the spot images were displayed at the chromosomal locations of the relevant clones to provide a visual comparison of DNA amplification sites with chromosomal locations of overexpressed genes. The chromosomal locations of the clones came from radiation hybrid-mapping data available in the human gene map *(21)*. Radiation hybrid mapping is a method of physical mapping that uses the frequencies of x-ray-induced DNA breakage to deduce distances between markers, which are expressed in centiRays (cR), the genetic map distance that corresponds to an interval in the radiation hybrid map in which there is a 1% probability of x-ray-induced DNA breakage.

Mapping and Copy Number Analysis of Ribosomal Protein S6 Kinase by Fluorescence *In Situ* Hybridization

A P1 artificial chromosome probe (clone 186o9) specific for ribosomal protein S6 kinase (S6K) was obtained by screening of a commercially available library (Genome Systems, Inc., St. Louis, MO) by the use of the polymerase chain reaction. The S6K probe was fluorescently labeled with SpectrumOrange–dUTP by use of random priming and was hybridized to normal metaphase chromosomes to map the S6K gene as described previously *(13)*. The SpectrumOrange-labeled S6K probe was also used together with a SpectrumGreen fluorescently labeled probe for the centromere of chromosome 17 for copy number analysis by interphase fluorescence *in situ* hybridization (FISH) as described previously *(13)*. The hybridizations were evaluated by use of a Zeiss fluorescence microscope. Approximately 20 nonoverlapping nuclei with intact morphology (evaluated by use of the DAPI counterstain) were scored to determine the mean number of hybridization signals for each probe. Amplification was measured by a comparison of the number of signals produced by use of the probe for S6K with the number produced by use of the probe for the chromosome 17 centromere.

Tissue Microarray Analysis by FISH

For the tissue microarrays, the FISH protocol was changed according to the type of tissue fixation used. The ethanol-fixed tissue microarray was deparaffinized, denatured at 73 °C for 5 minutes in 2× SSC (i.e., 300 m*M* NaCl and 30 m*M* sodium citrate) containing 70% formamide, and dehydrated in an ethanol series (70% , 80%, and 100%). The forma-

lin-fixed tissue microarray was deparaffinized, immersed in 0.2 N HCl, incubated in 1 *M* sodium thiocyanate solution at 80 °C for 30 minutes, and immersed in a protease solution (0.5 mg/mL in 0.9% NaCl; Vysis, Inc.) for 10 minutes at 37 °C. These slides were then postfixed in 10% buffered formalin (Sigma Chemical Co.), air-dried, denatured at 73 °C for 5 minutes in 2× SSC containing 70% formamide, dehydrated in an ethanol series, incubated in 4 μg/mL proteinase K (Boehringer Mannheim Biochemicals) at 37 °C for 7 minutes, and dehydrated again as above. Both ethanol-fixed and formalin-fixed slides were hybridized with the SpectrumOrange-labeled S6K-specific probe and the SpectrumGreen-labeled chromosome 17 centromere probe overnight at 37 °C. An adjacent section from the tissue microarray was hybridized with a SpectrumOrange-labeled HER-2 probe (Vysis, Inc.). Specimens containing tight clusters of signals or more than a threefold increase in the number of S6K signals, as compared with chromosome 17 centromere signals, in at least 10% of the tumor cells were considered to be amplified.

Our tissue microarrays comprised 984 tumor samples. Analysis of S6K and HER-2 copy numbers was uninformative in 316 and 374 cases, respectively, because of missing or unrepresentative tumor samples in the array section or failure of the analysis.

Northern Blot Analysis

Total RNA was extracted from breast cancer cell lines by use of the RNeasy kit. RNA (10 μg) was subjected to electrophoresis through a 1 *M* formaldehyde–1% agarose gel and transferred onto a Nytran membrane (Schleicher & Schuell, Inc., Keene, NH) by standard methods *(24)*. The insert from a sequence-verified cDNA clone (GenBank accession number H04661) representing the 3′-end of the S6K gene was ^{32}P-labeled by random priming (Prime-It; Stratagene Cloning Systems, La Jolla, CA). The blot was prehybridized with 10 μg/mL boiled, sheared DNA (Research Genetics, Huntsville, AL) for 1 hour at 68 °C in Express Hybridization solution (Clontech Laboratories, Inc.). Hybridization was done in the prehybridization solution at 68 °C overnight. The blot was washed several times with 1% SDS in 2× SSC at 65 °C and then in 0.5% SDS in 0.1× SSC (i.e., 15 m*M* NaCl and 1.5 m*M* sodium citrate) at 55 °C. Hybridized probe was detected by autoradiography. After removal of bound S6K probe, the membrane was rehybridized with a probe for glyceraldehyde-3-phosphate dehydrogenase messenger RNA (mRNA) to confirm equal loading among samples. The expression levels were quantitated by use of the PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

Western Blot Analysis

Breast cancer cell line monolayers were trypsinized to yield a cell suspension and centrifuged at 200*g* for 8 minutes at room temperature. The precipitated cells were lysed in RIPA buffer (i.e., 1% phosphate-buffered saline [PBS], 1% Nonidet P-40, 0.5% sodium deoxycholate, and 0.1% SDS) by repeated passage through a 21-gauge needle and centrifuged at 10 000*g* for 10 minutes at 4 °C. Approximately 40 μg of total protein was boiled in loading buffer (Laemmli Sample Buffer; Bio-Rad Laboratories, Hercules, CA) for 5 minutes, subjected to electrophoresis through a 4%–12% polyacrylamide gradient Tris-glycine gel (Novex, San Diego, CA), along with Full-Range Rainbow Molecular Weight Markers (Amersham Life Science, Little Chalfont, Buckinghamshire, U.K.), and electroblotted onto a nitrocellulose membrane (Novex). Detection of protein was by the BM Chemiluminescence Western Blotting Kit (Mouse/Rabbit) (Boehringer Mannheim Biochemicals). Briefly, the membrane was incubated for 1 hour at room temperature in 1% blocking solution (Boehringer Mannheim Biochemicals) and for 1 hour at 4 °C in a solution of 4 μg/mL primary antibody (anti-p70^{S6k} antibody; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) in 0.5% blocking solution. The membrane was washed two times for 10 minutes each in TBS–Tween® 20 (Boehringer Mannheim Biochemicals) and then twice with 0.5% blocking solution, incubated for 30 minutes at 4 °C with peroxidase-labeled secondary antibody (mouse anti-rabbit immunoglobulin G; Boehringer Mannheim Biochemicals), and then washed four times for 15 minutes each with large volumes of TBS–Tween® 20. Signal detection was done according to the manufacturer's instructions. The membrane was restained with anti-tubulin antibody to confirm equal loading of samples.

Measurement of S6K Activity

Breast cancer cell line monolayers in 100-mm dishes were washed once in PBS. The cells were lysed directly on the dishes by incubation for 20 minutes in 1 mL of cold modified radioimmunoprecipitation assay buffer (i.e., 50 m*M* Tris–HCl [pH.7.4], 1% Nonidet P-40, 0.25% sodium deoxycholate, 150 m*M* NaCl, 1 m*M* ethylene glycol-bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid, 1 m*M* phenylmethylsulfonyl fluoride, 1 m*M* Na_3VO_4, and 1 m*M* NaF) containing a protease inhibitor cocktail (Complete Protease Inhibitor Cocktail) prepared according to the manufacturer's instructions (Boehringer Mannheim Biochemicals). The lysates were passed repeatedly through a 21-gauge needle and centrifuged at 10 000*g* for 10 minutes at 4 °C, and the supernatant was collected. Approximately 200 μg of protein was incubated overnight at 4 °C with 1 μg of anti-p70^{S6k} antibody. The immunocomplex was absorbed to 50 μL protein A–agarose (Life Technologies, Inc.) by incubation for 3 hours at 4 °C and washed four times with cold PBS. S6K enzyme activity was assayed by use of the S6 Kinase Assay Kit according to the recommendations of the manufacturer (Upstate Biotechnology, Lake Placid, NY). Briefly, the agarose beads were suspended in 20 μL of assay dilution buffer, 10 μL of substrate peptide (500 μ*M* peptide AKRRRLSSLRA), 10 μL of inhibitor mixture, and 10 μL of radioactive adenosine triphosphate (ATP) mixture (i.e., 75 m*M* $MgCl_2$, 500 μ*M* ATP, and 10 μCi of [γ-^{32}P]ATP) and incubated for 10 minutes at 30 °C. Aliquots of 25 μL were spotted onto P81 phosphocellulose paper squares (Upstate Biotechnology) and washed three times with 0.75% phosphoric acid and once with acetone to remove unreacted [γ-^{32}P]ATP. The amount of radioactive product, which remains absorbed to the paper squares, was measured in an LS6500 scintillation spectrometer (Beckman Coulter, Inc., Fullerton, CA).

Growth Inhibition by Rapamycin

We plated 10^4 cells into each well of 96-well microtiter plates. The next day, serial dilutions of rapamycin (Calbiochem Corp., La Jolla, CA) were added. After incubation at 37 °C for 70 hours, the medium was replaced with fresh drug-free medium, and the cells were allowed to recover overnight. Growth inhibition was measured by use of the colorimetric MTT (i.e., 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrasodium bromide) test (Chemicon International, Inc., Temecula, CA). All experiments were performed in triplicate, and the results were expressed as percent inhibition relative to control cultures without rapamycin.

Immunohistochemistry

The expression of S6K in primary breast tumors was analyzed by the use of immunohistochemistry to the tissue microarray that contained 612 paraffin-embedded primary breast cancer specimens. Analysis of 167 was uninformative because of missing or unrepresentative tumor samples in the array section or failure of the analysis. A standard indirect immunoperoxidase protocol with 3,3′-diaminobenzidine as a chromogen was used for immunohistochemistry (ABC-Elite; Vector Laboratories, Inc., Burlingame, CA). Polyclonal anti-p70^{S6k} antibody was used for detection of S6K (1 : 5000 dilution in PBS containing 1% bovine serum albumin and 0.1% Triton X-100). A high-temperature (20 minutes in a pressure cooker) treatment procedure with Antigen Unmasking Solution (Vector Laboratories, Inc.) was used to enhance the staining. The primary antibody was omitted for negative controls. S6K staining of cytoplasm was subjectively scored into four groups: negative (no staining), weak, moderate, or strong staining. For statistical analyses, the data were combined into two groups: low expression (negative or weak staining) and high expression (moderate or strong staining).

Statistical Analyses

The relationship of S6K amplification to clinicopathologic parameters and the association between S6K amplification and S6K expression were analyzed by use of Fisher's exact test. The survival curves were estimated by Kaplan–Meier analysis. The prognostic significance of amplification of S6K and HER-2 was analyzed by use of the log-rank test. All *P* values are two-sided.

RESULTS

cDNA Microarray Analyses

Our search for amplified DNA sequences in breast cancer was based on results from CGH, which maps amplified DNA sequences on metaphase chromosomes. In CGH, fluorescently labeled test and control DNAs are hybridized to normal metaphase chromosomes, and the fluorescence intensities and their ratios are quantitated.

One of the most prominent amplification sites in the MCF-7 cells is localized at 17q23 (Fig. 1, A and B). We used a cDNA microarray that contained 4209 clones to evaluate expression levels in MCF-7 cells relative to those in normal mammary epithelial cells. To better visu-

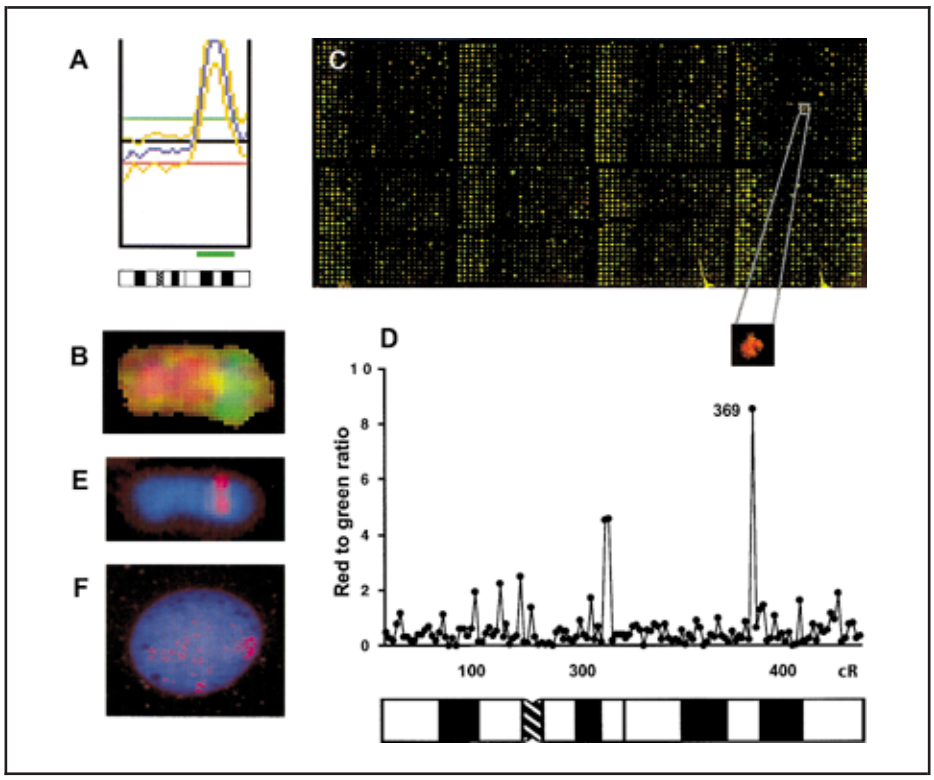

Fig. 1. Comparison of complementary DNA (cDNA) microarray data on the MCF-7 breast cancer cell line with comparative genomic hybridization (CGH) results. **Panel A:** CGH copy number ratio profile for chromosome 17. Normal metaphase chromosomes were hybridized with fluorescently labeled MCF-7 DNA and normal placental DNA. The fluorescence intensities were measured along chromosome 17, and their ratios (DNA sequence copy number in MCF-7 cells/copy number in normal tissue) were calculated. The average copy number ratio from 10 chromosomes, aligned with the chromosome ideogram, is shown in **blue,** with 95% confidence intervals in **orange.** The **horizontal black line** represents a ratio of 1.00; **green,** a ratio of 1.15; and **red,** a ratio of 0.85. The **green bar at the bottom of the figure** indicates the region of chromosome 17 with increased copy number in MCF-7 cells. **Panel B:** A computer-generated image of chromosome 17 from a CGH experiment of MCF-7 cells, aligned with the chromosome 17 ideogram and graph in panel A. Fluorescent images corresponding to the MCF-7 DNA and placental DNA were acquired separately by the use of a Zeiss fluorescence microscope, displayed in pseudo-colors, and overlaid. The **green color** indicates amplification of MCF-7 DNA at the corresponding location in the chromosome. The **red color** indicates hybridization from the placental DNA. **Panel C:** A computer-generated cDNA microarray image of 4209 clones. The microarray was hybridized separately with Cyanine5–deoxyuridine triphosphate (dUTP)-labeled MCF-7 cDNA and Cyanine3–dUTP-labeled normal mammary cDNA as described in the "Methods" section. The fluorescent images from the two hybridizations were recorded separately with a confocal microscope. The MCF-7 cDNA image was assigned a **red color** and the normal mammary cDNA image a **green color** to form a pseudo-color image. **Spots that appear red** denote clones that were more highly expressed in MCF-7 cells than in normal breast tissue, and **spots that appear green** denote clones that were more highly expressed in normal breast tissue than in MCF-7 cells. **Inset:** an enlarged image of the spot that had the highest red-to-green ratio of all clones on the array. **Panel D:** Distribution of expression ratios for genes along chromosome 17. The **red to green ratios** for 135 genes mapping to chromosome 17 were calculated from a cDNA microarray (panel C) and are shown in alignment with the chromosome 17 ideogram from 17pter to 17qter according to their radiation hybrid map positions in centiRays (cR). The peak at 369 cR corresponds to the enlarged inset image in panel C (highest level of expression of all clones on the array). **Panel E:** Fluorescence *in situ* hybridization (FISH) of the SpectrumOrange-labeled DNA probe for ribosomal protein S6 kinase (S6K) to normal chromosome 17. The image was produced by the use of a Zeiss fluorescence microscope equipped with a 63× objective. The **red color** indicates the specific hybridization signals at 17q23, and the **blue color** indicates the chromosomal DNA counterstain. The image is aligned with those in panels A and B. **Panel F:** FISH of the SpectrumOrange-labeled DNA probe for S6K to MCF-7 interphase nucleus. The image was produced as in panel E. The **red color** indicates the specific hybridization signals of the S6K probe, and the **blue color** indicates the nuclear DNA counterstain.

alize the relationship between increased copy number and increased expression levels of genes along chromosome 17, we displayed the data from our cDNA microarray analyses according to the clones' chromosomal positions.

Fig. 1 illustrates the comparison of a CGH copy number ratio profile for chromosome 17 (panel A) with the distribution of the expression ratios for genes along chromosome 17, arranged from 17pter to 17qter (panel D). The largest peak in the cDNA microarray profile (Fig. 1, D) corresponds to an expressed sequence tag (EST) (image clone identification No. 133432). This clone showed a higher MCF-7-to-normal expression ratio (8.4) than any other evaluable cDNA clone in the cDNA microarray experiment (Fig 1, C, and inset). This EST belongs to a UniGene cluster Hs.124943 *(25)* located at 17q23 by radiation hybrid mapping (at 369 cR) *(21).* Subsequent sequencing and extension of the cDNA clones in this UniGene cluster (Forozan F, Kallioniemi O-P: unpublished observations) indicated that this UniGene represented the 3′ untranslated region of the ribosomal protein S6K. The localization of S6K to 17q23 was confirmed by FISH to normal metaphase chromosomes (Fig. 1, E). Since S6K is an important signaling molecule involved in the transition from G_1 to S phase of the cell cycle, we explored further the role of S6K as a possible amplification target gene.

S6K Copy Number, Expression, and Enzymatic Activity in Breast Cancer Cell Lines

The S6K gene copy number was analyzed in seven breast cancer cell lines by interphase FISH. Cell line MCF-7 showed at least a 10-fold amplification (Fig. 1, F), as did cell lines KPL-1 and ZR-75-1wt (data not shown). In other experiments for which the data are not shown, cell line Sum-52 showed a twofold amplification, cell line BT-474 showed at least a fivefold amplification, and cell lines HBL-100 and MDA-436 showed no increase in S6K copy number.

Expression of S6K mRNA and protein was studied by northern and western blot analyses. Northern blot analysis revealed major transcripts with molecular sizes of 2.5 and 6.0 kilobases (Fig. 2, A), consistent with the results of Reinhard et al. *(26).* In the four cell lines that showed at least a fivefold increase in S6K copy number, expression of both of these transcripts was increased eightfold to 20-fold (relative to cell lines with no amplification). Western blot analysis of five cell lines revealed bands with apparent molecular masses of 70 and 85 kilodaltons, which correspond to the two known isoforms of S6K protein *(27).* Increased expression of both isoforms was seen in

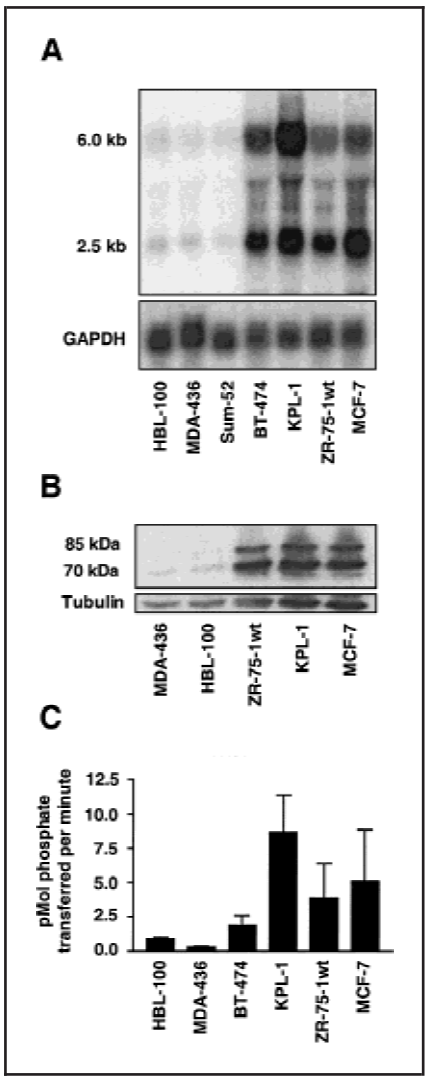

Fig. 2. Ribosomal protein S6 kinase (S6K) messenger RNA (mRNA) and protein expression and kinase activity in breast cancer cell lines. **Panel A:** Total RNA from breast cancer cell lines HBL-100, MDA-436, Sum-52, BT-474, KPL-1, ZR-75-1wt, and MCF-7 was analyzed for expression of ribosomal protein S6K mRNA by northern blot analysis as described in the "Methods" section. The sizes (in kilobases, kb) of the major transcripts (2.5 kb and 6.0 kb) were estimated by comparison to a size standard. The membrane was rehybridized with a probe complementary to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA as a control to confirm equal loading of RNA in all seven lanes. **Panel B:** Expression of S6K protein by cell lines MDA-436, HBL-100, ZR-75-1wt, KPL-1, and MCF-7 was measured by western blot analysis by use of anti-$p70^{S6k}$ antibody as described in the "Methods" section. The sizes (in kilodaltons, kDa) of the major products (70 kDa and 85 kDa) were estimated by comparison to a size standard. The membrane was restained with anti-tubulin antibody as a control for equal loading of protein in all five lanes. **Panel C:** S6K activity in breast cancer cell lines HBL-100, MDA-436, BT-474, KPL-1, ZR-75-1wt, and MCF-7. Cell lysates were incubated with anti-$p70^{S6k}$ antibody, and the immunocomplex was adsorbed to protein A–agarose and assayed for phosphotransferase activity (pMol phosphate transferred per minute) as described in the "Methods" section. **Error bars** are 95% confidence intervals.

three cell lines with S6K amplification (Fig. 2, B).

The S6K protein is activated by phosphorylation on multiple sites *(28,29).* We, therefore, studied whether the increased expression of S6K protein would also lead to increased enzymatic activity. When compared with the average enzymatic activity in lysates of HBL-100 and MDA-436 (cell lines with neither amplification nor overexpression), the activity observed in lysates from four cell lines with S6K amplification and overexpression ranged from threefold (cell line BT-474) to 15-fold (cell line KPL-1) (Fig. 2, C).

Growth inhibition by rapamycin, which blocks the phosphorylation of S6K *(28),* was studied in six cell lines. Over the range of rapamycin concentrations used (0.01–100 n*M*), all cell lines showed concentration-dependent growth inhibition, regardless of their amplification status (data not shown).

S6K Amplification and Protein Expression in Primary Breast Cancers by Tissue Microarrays and Clinicopathologic Associations

We applied FISH analysis on tumor tissue microarrays to evaluate the amplification of the S6K gene *in vivo* in 668 informative primary breast tumors (Fig. 3, A). Fifty-nine tumors (8.8%) showed amplification of S6K (Fig. 3, B). The S6K protein expression was analyzed in a subset of 445 tumors by use of immunohistochemistry. Fig. 3, C and D, illustrates tumor samples with no S6K expression or high S6K expression, respectively. Moderate or strong cytoplasmic staining was seen in 69 primary tumors (15.6%). There was a statistically significant association between S6K amplification and high S6K expression ($P = .0004$), with 41.2% (14/34) of the amplified tumors exhibiting high S6K expression.

No statistically significant association was seen between S6K amplification and the age of the patient, tumor size, lymph node status, histologic grade, or the presence of estrogen or progesterone receptors (data not shown). However, patients with S6K amplification had a statistically significantly ($P = .0021$) worse survival than patients without S6K amplification (Fig. 4, A). A similar, statistically significant ($P = .0083$) prognostic relationship was also found with high S6K expression (Fig. 4, B).

Since amplification of the HER-2 oncogene (located on the same chromosome at 17q12) is also associated with poor prognosis in breast cancer *(30),* we analyzed whether S6K showed prognostic significance irrespective of HER-2. An adjacent section from the tissue microarray was hybridized with a probe for HER-2, which showed amplification in 106 (17.4%) of 610 informative cases. Amplification of both S6K and HER-2 was seen in 27 (4.4%) of the 610 cases, whereas S6K was amplified alone in 30 cases (4.9%). Patients with tumors showing both S6K and HER-2 amplifications had a statistically significantly ($P = .0001$) worse prognosis than those with no amplification or amplification of only one of the genes (Fig. 4, C).

Discussion

Genomic DNA amplifications are common in breast cancer and affect a number of distinct chromosomal sites. At each amplification site, multiple genes may confer a clonal growth advantage to the cancer cells. Such genes could be promising targets for developing diagnostic, prognostic, and therapeutic approaches for breast cancer. This concept has been best demonstrated for the HER-2 gene [*see (30)* for a recent review], which originally was found to be amplified in neuroblastomas and subsequently was shown to be associated with poor prognosis in breast cancer. More than 10 years after these initial discoveries, treatment of HER-2-positive breast cancers with the monoclonal antibody Herceptin is now available *(30).*

To rapidly identify putative target genes for other genomic amplifications detected in cancer by CGH, we performed a parallel analysis of gene expression changes by use of cDNA microarrays in the MCF-7 breast cancer cell line and normal mammary gland tissue. The sensitivity of our cDNA microarray approach has been described in detail previously

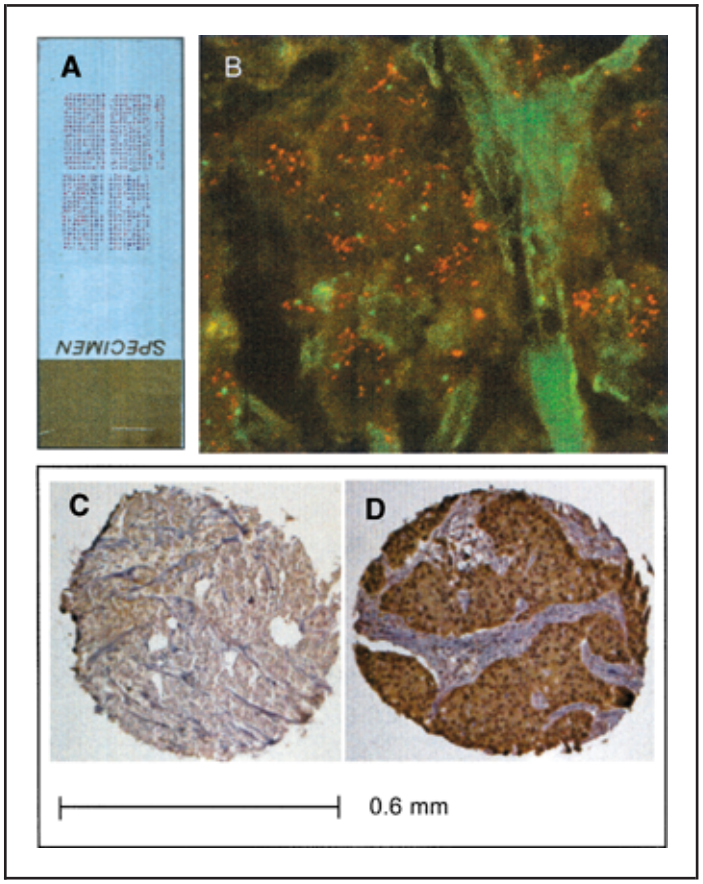

Fig. 3. Analysis of ribosomal protein S6 kinase (S6K) amplification and expression in primary breast tumors. **Panel A:** A hematoxylin–eosin-stained section from a tissue microarray containing 612 primary breast cancer specimens. **Panel B:** Fluorescence *in situ* hybridization analysis. The tissue microarray was hybridized with a SpectrumOrange-labeled ribosomal protein S6 kinase (S6K)-specific probe and a SpectrumGreen-labeled probe for the chromosome 17 centromere, as described in the "Methods" section. The image of a representative tissue sample was produced by the use of a Zeiss fluorescence microscope equipped with a 63× objective. The **red spots** indicate the S6K-specific hybridization signals, and the **green spots** indicate the chromosome 17 centromere-specific hybridization signals. **Panels C and D:** Immunohistochemical detection of S6K protein. The tissue microarray was incubated with polyclonal anti-$p70^{S6k}$ antibody, as described in the "Methods" section to give a brown precipitate that corresponds to the presence of S6K protein. **Panel C:** one representative tissue sample that shows no expression of S6K. **Panel D:** one representative tissue sample that shows high-level expression of S6K. The **scale bar** indicates the diameter of the tissue samples.

(18,22). In earlier experiments, we were able to detect overexpression of several genes shown to be expressed in MCF-7, including CDK4, CAS, MYBL, and PCNA (Forozan F, Mahlamäki EH, Monni O, Chen Y, Veldman R, Jiang Y, et al.: unpublished data). In separate control experiments, we have also been able to reliably detect expression of rare transcripts, such as that of the estrogen receptor (data not shown).

The most highly overexpressed gene in the MCF-7 cells was an EST corresponding to the ribosomal protein S6K, a gene mapping to 17q23, a chromosomal region that harbors the most prominent amplification site in the MCF-7 cell line. Based on its biologic role, S6K represents an ideal candidate for a gene whose activation by amplification could contribute to oncogenesis. The S6K protein is rapidly activated in response to various mitogenic stimuli, such as growth factors (e.g., epidermal growth factor and platelet-derived growth factor), cytokines, and oncogene products *(28,31,32).* S6K regulates protein synthesis, translation of specific mRNA species, and progression from the G_1 to the S phase of the cell cycle *(28,32,33).*

We observed high-level amplification of the S6K gene in all four of the cell lines with 17q23 amplifications that we examined. We also observed that the levels of mRNA and protein expression were often higher than those anticipated from the level of DNA amplification and that S6K activity was increased in all cell lines that showed S6K gene amplification. These observations support the hypothesis that the S6K gene is an amplification target gene in breast cancer cell lines.

Although 17q23 amplifications are detected by CGH in both breast cancer cell lines and uncultured primary breast tumors *(4–7),* it is important to validate any data from cell line model systems in clinical specimens. The tissue microarray technology *(14)* provides an ideal method for rapid *in vivo* analysis of candidate cancer genes in a large series of patient specimens. S6K gene amplification and protein expression were seen in 8.8% and 15.6%, respectively, of breast cancer specimens. Statistically significant associations were found between gene amplification or protein expression and poor prognosis of patients. These results suggest that S6K amplification is characteristic of aggressive breast cancers. In more than half of the tumors, S6K was amplified irrespective of HER-2, a known indicator of poor prognosis in breast cancer *(30).* Co-amplification of both of these genes implied a much worse prognosis than did amplification of either one alone.

A recent study *(34)* also supports the role of S6K as a possible target gene for amplification of 17q23 in breast cancer. Couch et al. *(34)* found amplification of S6K in two of 14 patient specimens evaluated. Our results, which are based on samples from a large number of patients, indicate that additional clinical studies are warranted to explore the role of S6K amplification both as an independent prognostic indicator and as a modifier of disease progression (such as response to Herceptin therapy) in patients with tumors in which HER-2 is amplified.

Inhibition of the increased S6K activity in cancer cells with 17q23 amplification could provide an attractive target for therapeutic intervention. Our results indicate that growth inhibition by rapamycin, a known inhibitor of S6K activity, was not appreciably different between cell lines with increased S6K activity and those with no S6K involvement. This observation may indicate that rapamycin is not a sufficiently specific inhibitor, since it also influences a wide variety of other cellular functions such as induction of apoptosis and inhibition of several cyclins

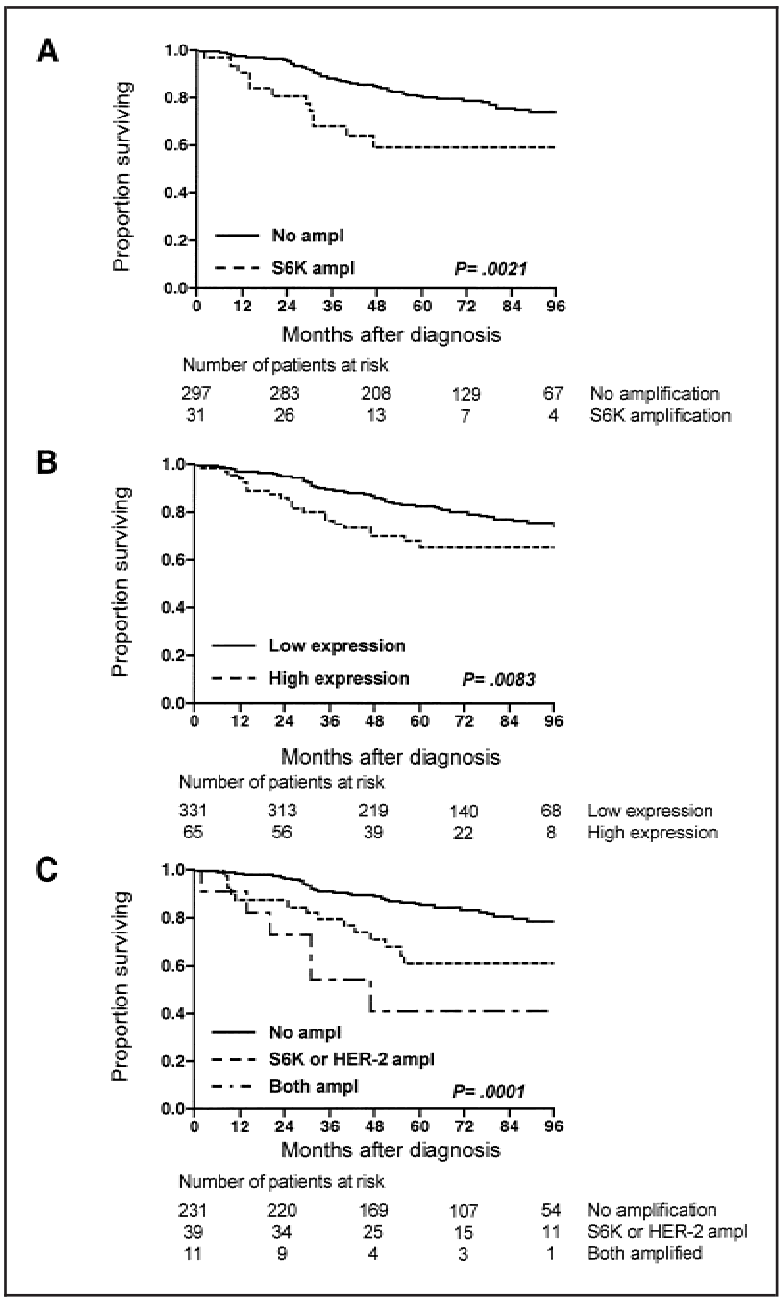

Fig. 4. Kaplan–Meier analysis of breast cancer-specific survival. **Panel A:** Survival according to amplification (ampl) of ribosomal protein S6 kinase (S6K). At 2 years after diagnosis, breast cancer-specific survival was 80.6% (95% confidence interval [CI] = 65.4%–95.8%) for patients with S6K amplification and 95.6% (95% CI = 93.2%–98.0%) for patients without S6K amplification. At 4 years, breast cancer-specific survival was 59.2% (95% CI = 32.5%–85.9%) for patients with S6K amplification and 84.6% (95% CI = 79.7%–89.5%) for patients without S6K amplification. At 6 years, breast cancer-specific survival was 59.2% (95% CI = 22.8%–95.6%) for patients with S6K amplification and 78.4% (95% CI = 71.3%–85.5%) for patients without S6K amplification. **Panel B:** Survival according to S6K protein expression. At 2 years after diagnosis, breast cancer-specific survival was 86.0% (95% CI = 76.9%–95.1%) for patients with high S6K expression and 95.1% (95% CI = 92.7%–97.5%) for patients with low S6K expression. At 4 years, breast cancer-specific survival was 69.9% (95% CI = 55.5%–84.3%) for patients with high S6K expression and 86.1% (95% CI = 81.5%–90.7%) for patients with low S6K expression. At 6 years, breast cancer-specific survival was 65.4% (95% CI = 45.5%–85.3%) for patients with high S6K expression and 79.7% (95% CI = 73.0%–86.4%) for patients with low S6K expression. **Panel C:** Survival according to amplification of S6K and HER-2. At 2 years after diagnosis, breast cancer-specific survival was 72.7% (95% CI = 43.6%–100%) for patients with amplification of both S6K and HER-2, 87.2% (95% CI = 76.0%–98.4%) for patients with either S6K or HER-2 amplification, and 96.5% (95% CI = 94.1%–98.9%) for patients with no amplification. At 4 years, breast cancer-specific survival was 40.9% (95% CI = 0%–89.1%) for patients with amplification of both S6K and HER-2, 71.2% (95% CI = 53.4%–89.0%) for patients with either S6K or HER-2 amplification, and 88.7% (95% CI = 83.9%–93.5%) for patients with no amplification. At 6 years, breast cancer-specific survival was 40.9% (95% CI = 0%–96.5%) for patients with amplification of both S6K and HER-2, 61.1% (95% CI = 36.4%–85.8%) for patients with either S6K or HER-2 amplification, and 83.0% (95% CI = 75.9%–90.1%) for patients with no amplification. **Below each graph** is the total number of patients at risk at various time points. Two-sided *P* values were calculated by the log-rank test.

(28,35–37). Therefore, studies with more specific inhibitors of S6K activity are warranted. It is also possible—and even likely—that S6K is not the only gene affected by the 17q23 amplifications and that simultaneous activation of several genes at 17q23 could contribute to cancer progression. Our cDNA microarray analysis was done on 4209 clones that did not represent a complete set of genes from a particular chromosomal region. The use of focused cDNA microarrays that include more genes from the very gene-rich 17q23 region will allow a more comprehensive analysis of the molecular consequences of the 17q23 amplifications and perhaps highlight additional overexpressed genes.

Our results illustrate how the combination of CGH information with cDNA and tissue microarray analyses can be used to identify amplified and overexpressed genes and to evaluate the clinical implications of such genes and genomic rearrangements. cDNA microarrays are now widely used to identify differentially expressed genes in human cancer *(38).* CGH information on genomic copy number provides a new parameter to take into account when interpreting gene expression information from thousands of cDNA clones and may help to identify the most important targets first. Focused high-throughput analysis of hundreds of clinical samples by use of tissue microarray technology complements these genome-screening tools and provides information on genes that are likely to be important for cancer development and progression in patients.

Our analyses imply that the S6K gene is likely to be an important—but perhaps not the only—target gene involved in the 17q23 amplifications in breast cancer. S6K amplification is characteristic of aggressive types of breast cancer, and detection of S6K amplification in conjunction with HER-2 amplification may provide more prognostic information than either one alone. Further research is required both on the role of S6K and on the possible presence of other overexpressed genes in this amplicon.

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NOTES

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