Localization of \(PS6K\) to Chromosomal Region 17q23 and Determination of Its Amplification in Breast Cancer\(^1\)

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Abstract

The application of comparative genomic hybridization to the analysis of genetic abnormalities in breast carcinoma has consistently revealed that chromosome region 17q22–24 is a frequent site of gene amplification in this type of cancer. As part of an examination of expressed sequence tags for novel amplified genes in this region, we identified \(PS6K\) amplifications in both breast tumor tissues and cell lines. \(PS6K\) was localized to 17q23 and encodes a serine-threonine kinase whose activation is thought to regulate a wide array of cellular processes involved in the mitogenic response including protein synthesis, translation of specific mRNA species, and cell cycle progression from G1 to S phase. Northern and Western analyses revealed that amplification of this gene was accompanied by corresponding increases in mRNA and protein expression, respectively. These data represent the first determination of a gene amplification within 17q22–24 in breast cancer and suggest an oncogenic activity for \(PS6K\).

Introduction

Gene amplification is a common event in breast cancer, and it has been shown to involve multiple loci at various chromosomal locations. Of these, the long arm of chromosome 17 is the most frequently affected, and it contains independently amplified regions at q21 (1, 2) and q22–24 (3–5). \(ERBB2\) is located at 17q21 and is well accepted as the target gene for amplifications within this region, although additional amplified sequences have been identified at this chromosomal location (6). Specific genes involved in q22–24 amplifications have yet to be identified, and until recently, these alterations had only been demonstrated by comparative genomic hybridization (3, 4), a technique with limited resolving power.

There are, however, several approaches that one may take to the identification of amplified genes once a cytogenetic location has been established for an amplicon of interest. One that we have recently used (7) involves the utilization of databases containing regionally localized ESTs.\(^3\) By synthesizing ESTs and applying them as probes to the Southern analysis of appropriate tumor DNAs, it is possible to identify amplified genes as well as to construct ampiclon maps. By using this approach for the analysis of the 17q22–24 region in breast cancer, we have identified two amplified and overexpressed sequences. One of the ESTs is associated with a known gene, \(PS6K\). In mammalian cells, the protein product of \(PS6K\), s6k, is thought to activate the translation initiation machinery in response to growth factor stimulation of its upstream regulator, TOR (8), and s6k activity has been shown to be necessary for entry into S phase of the cell cycle (9, 10). In combination with these functional properties of s6k, our genetic data suggest an oncogenic activity for \(PS6K\) and indicate the existence of additional chromosome 17q amplification targets.

Materials and Methods

Tumor Tissues and Cell Lines. All tumors used in this study were obtained from patients undergoing surgical treatment at the Mayo Clinic (Rochester, MN) and were diagnosed as primary stage 2 breast carcinomas. The breast carcinoma cell lines examined, MDA-157, UACC-893, MCF-7, BT-474, UACC-812, and MDA-361, were obtained from American Type Culture Collection.

Nuclear Acid Extraction and Analysis. Cell line and tumor DNAs were isolated as described previously (11). DNAs were treated with HindIII restriction enzyme, electrophoresed through 0.8% agarose gels, transferred to a reinforced nitrocellulose membrane (Nytran; Schleicher and Schuell, Keene, NH), and fixed to the membrane by baking in a vacuum oven at 80°C for 2 h.

RNA extractions from cell lines were performed with a modification of the technique described by Chomczynski and Sacchi (12), in which pellets were lysed in a guanidinium thiocyanate-phenol solution (Trizol; Life Technologies, Inc.) by vortexing. After the addition of one-fifth volume of chloroform to sample lysates, specimens were vortexed again and centrifuged. Aqueous, RNA-containing phases were isolated from centrifuged specimens, and sample RNAs were precipitated with isopropanol and resuspended in DEPC-treated H2O. RNA samples were electrophoresed through formaldehyde-agarose gels (1.0%) and blot-transferred and fixed to nitrocellulose membranes as described above.

DNA and RNA filters were hybridized with \(^3\)P-labeled probes for \(PS6K\) (cDNA bases 1520–1695, synthesized by PCR of genomic DNA) and \(ERBB2\) (full-length cDNA coding sequence, bases 149–3955). After hybridization, filters were washed and exposed to X-ray film; treatments of DNA and RNA filters for multiple, sequential hybridizations have been described previously (11, 13).

Protein Isolation and Western Analysis. Breast tumor cell line pellets were swelled in cold PBS at room temperature for 15 min and lysed by repeated passage through a 27-gauge needle. Lysates were cleared of insoluble material by microcentrifugation at 13,000 rpm for 15 min at 4°C, and protein concentrations were determined (protein assay kit; Bio-Rad). Approximately 50 \(\mu\)g of total protein from each sample were boiled in loading buffer for 5 min, electrophoresed through 10% polyacrylamide gels, and electroblotted to a reinforced nitrocellulose membrane (Hybond-C extra; Amersham). The membrane was incubated overnight at 4°C in a blocking solution consisting of 0.1% Tween 20, 1× Tris-buffered saline, and 5% nonfat dry milk. Incubation with primary antibody (rabbit antihuman s6k: Santa Cruz Biotechnology) was performed for 2 h at room temperature in blocking solution. After incubation with primary antibody, filters were washed repeatedly in PBS with 0.1% Tween 20 at room temperature and then incubated in blocking solution for 1 h at room temperature with horseradish peroxidase-labeled secondary antibody (goat antirabbit IgG; BMB). Signal detection of filter-bound secondary antibody was by horseradish peroxidase chemiluminescent reaction (ECL; Amer sham).

FISH Analysis. Hybridizations of a SpectrumGreen-labeled chromosome 17 centromeric probe (Vysis, Downers Grove, IL) and a SpectrumOrange-labeled \(PS6K\) probe (isolated from the RPCI-11 Human BAC Library; Research Genetics) were performed on slides containing either normal or MCF-7 metaphase chromosomes. Metaphase chromosomes were prepared for hybridization by heating at 65°C for 1 h followed by a 37°C incubation in 2× SSC for 1 h followed by a 1 min incubation in 70% formamide at 72°C.

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\(^3\)The abbreviations used are: EST, expressed sequence tag; FISH, fluorescence in situ hybridization; BAC, bacterial artificial chromosome.
some 17 centromere and PS6K probes that had been heated at 74°C for 5 min were applied to the denatured metaphase chromosomes and incubated at 37°C overnight. Posthybridization washes were performed in 0.4 × SSC at 72°C for 3 min, followed by 4 × SSC-0.1% Tween 20 for 5 min at room temperature and then 5 min in 2 × SSC at room temperature. Hybridization signals were detected using a Zeiss Axioplan microscope equipped with a triple-pass filter (4′,6-diamidino-2-phenylindole/Green/Orange: Vysis).

Results

Cell lines previously determined to have one or more regions of 17q amplification (5) were examined by Southern analysis using several EST probes localized to the q22–24 region (Table 1). Two of these, WI-6034 and WI-6857, showed normalized dosage increases of >4-fold in three of the cell lines (MCF-7, BT-474, and MDA-361; Fig. 1A). When the same filter was rehybridized with an ERBB2 probe, increased signal response was evident in BT-474 and MDA-361 but not in MCF-7 DNA.

The WI-6034 EST was determined to be part of the PS6K gene, and the use of this EST as a probe for Northern analysis revealed two transcripts (Fig. 1B), results consistent with previously published data on PS6K expression (14). As was the case for WI-6034, hybridization of WI-6857 revealed overexpressed transcripts (data not shown) in the cell lines in which it is amplified. This latter EST is not associated with a known gene. Expression levels for the protein product of PS6K,

Table 1  EST amplification patterns in breast tumor cell lines

<table>
<thead>
<tr>
<th>EST</th>
<th>MCF-7</th>
<th>BT-474</th>
<th>MDA-361</th>
<th>MBa</th>
</tr>
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<tbody>
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<td>–</td>
<td>+</td>
<td>–</td>
<td>44.4</td>
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<tr>
<td>WI-6277</td>
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<td>–</td>
<td>–</td>
<td>57.9</td>
</tr>
<tr>
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<td>59.4</td>
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<td>–</td>
<td>–</td>
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<td>+</td>
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<tr>
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<td>–</td>
<td>–</td>
<td>64.1</td>
</tr>
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</table>

a MB, estimated distances (in Mb) from p-ter; Radiation Hybrid Consortium.

b The ERBB2 probe was a full-length cDNA.
s6k, were detected in cell lines by Western blot analysis. The results showed predominant expression of the p70 isoform (14, 15) and, as would be expected based upon the Southern analysis, s6k expression was highly elevated in cell lines with PS6K amplification (Fig. 1C).

To determine whether PS6K amplification occurs in primary tumors, we examined 14 breast carcinoma DNAs for increased dosage of the WI-6034 EST (Fig. 1D). Amplified PS6K was evident in two of these cases, and in one instance, it was apparent that its amplification occurred without the amplification of ERBB2. Elevated s6k expression was also evident in tumors with amplified PS6K (data not shown).

To confirm the genomic location of PS6K, we performed FISH analysis on normal metaphase chromosomes using a BAC clone identified from the RPCI-11 Human BAC Library using primers for the WI-6034 EST. Surprisingly, two regions of 17q homology were revealed by this analysis: one near the centromere and a second more distal in the expected q22–24 region (Fig. 2A). Comparison of FISH signal locations against corresponding G-banded chromosome images showed that the distal signal was located in band q23. Simultaneous hybridization of ERBB2 and PS6K probes revealed that the ERBB2 gene was located between the two signals associated with the PS6K probe and was, in fact, much nearer to the centromere-proximal PS6K homology region (data not shown). Hybridization of the PS6K BAC to MCF-7 cells also failed to provide a definitive chromosomal location of the PS6K amplicon as amplified regions were observed on several rearranged marker chromosomes (Fig. 2B).

To determine which of the 17q locations contained PS6K, we collected two additional pieces of data. The first of these was obtained from the Stanford radiation hybrid G3 mapping panel, which was used for localizing WI-6034. The results from this analysis clearly positioned the EST within the distal 17q region based upon its centiRay map location (2517 cR, estimated distance from p-ter = 61.4 Mb) and orientation relative to ERBB2 (1460 cR, estimated distance from p-ter = 45.5 Mb). In addition, WI-6034 has been localized to the 17.8 17q23 yeast artificial chromosome contig, generated and sequenced at the Whitehead Institute. The deduced PS6K exon-intron structure from this contig sequence is shown in Fig. 3 and reveals 15 exons extending over ~50 kb of genomic DNA. Collectively, these data indicate that the PS6K amplicon should reside within q23, and this location is consistent with the 17q amplification region assigned to MCF7 cells by comparative genomic hybridization analysis (5).

**Discussion**

In this study, we used a panel of mapped EST markers to examine a region of chromosome 17q for novel gene amplifications. This analysis led to the identification of two amplified and overexpressed sequences, one of which is associated with a gene encoding a protein that functions to promote cell proliferation (8). Because of its proximity to ERBB2, it is reasonable to question whether PS6K is only amplified as a result of its being a “bystander” to a well-documented oncogene; this appears to be the case for DDX1, which resides within a region that can be coamplified with MYCN in neuroblastoma (16, 17). We have shown, however, that PS6K amplification can occur independently of ERBB2, thereby establishing a circumstance similar to that observed for the independent or coamplifications of CDK4 and MDM2 that occur in chromosomal region 12q13–15 (18, 19). Despite its established role in the mitogenic response, it is far from clear whether PS6K represents a target gene for 17q amplifications in...
breast cancer. The results of this study only provide a starting place from which to begin a comprehensive analysis of the amplification region. Ultimately, the determination of amplification targets in 17q22–24 will rely on a combination of amplicon mapping and functional analyses of amplified genes.

An interesting aspect of this study concerns the identification of a duplicated region on 17q (Fig. 2A). Our examination of DNA sequence flanking the PS6K gene (BAC hRPK178_C_3) resulted in the identification of loci having >80% homology with the TRE-2 and TRE-17 genes, both of which have been localized to a centromere-proximal region on 17 (20). The amount of DNA encompassing these two genes is at least 60 kb, supporting the idea that a large segment of 17q has been duplicated. It is interesting to speculate that these duplicated regions may play a role in mediating the amplifications as well as deletions of 17q that occur in a large proportion of breast tumors; further analysis of these regions should provide some insight regarding this possibility.

References


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